The interaction of CD23 and CR2 and its functional consequences.

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The interaction of CD23 with CR2 and its functional consequences

by

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ABSTRACT

CD23/FcεRII is the low affinity receptor for IgE on B lymphocytes and the counterstructure for the complement receptor type 2, (CR2, CD21, EBV receptor); it is the interaction of CD23 with CR2 that has been the subject of this study. Recombinant CD23 fragment comprising the entire extracellular domain of human CD23 was expressed in *E. coli* and large amounts of the protein were obtained and purified. An optimal protocol for refolding of denatured inactive protein expressed in bacterial cells has been developed. The major molecular form of the recombinant soluble CD23 was found to be a dimer but there also appears to exist an equilibrium between dimer and trimer, as well as between monomer and dimer. A model has been proposed in which the transition of CD23 dimer into trimer occurs at the protein concentration of 10^{-5} M. It has been further demonstrated that the recombinant dimeric CD23 binds to CR2 on B cells and also interacts with recombinant soluble CR2 expressed in insect cells. Moreover, it has been shown that CD23 can crosslink IgE and CR2 *in vitro*. The functional consequence of CD23-CR2 interaction has been studied; CD23 expressed together with the low affinity receptor for IgG (CD32) in a fibroblast cell line was found to be stimulatory for tonsillar B cells in as much as it: (i) lowered the threshold for activation of cells by a factor of 10-100, (ii) enhanced the cellular response to antigen receptor ligation and (iii) modulated B cell response to CD19 ligation. In addition to the adhesion type interaction with CR2 that has been shown responsible for the stimulatory effects of CD23, there also appears to be a synergistic interaction between CD23 and CD32, the manner of which resembles the synergistic interaction of CR2 and CD19 with antigen receptor. Thus, CD23 must now be added to the list of CR2 ligands (C3d, EBV) and antibodies that can induce proliferation of B cells through this receptor.

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To my brother, *post mortem*.

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Abbreviations

AET: 2-aminoethylisothiouronium

BSA: Bovine serum albumin

CR: Complement receptor

CRD: Carbohydrate recognition domain

DioC18: 3,3'-dioctadedecyloxacarbocyanine perchlorate

EBV: Epstein-Barr virus

EDC: 1-ethyl-3-(dimethylaminopropyl)-carbodiimide-HCl

FBS: Foetal bovine serum

FDC: Follicular dendritic cells

GC: Germinal center

GnCl: Guanidine hydrochloride

HAT: Hypoxhantine/aminopterin/thymidine

HBS: Hepes buffered saline

HT: Hypoxhantine/aminopterin

IL-1: Interleukin-1

IL-4: Interleukin-4

IPTG: Isopropyl-β-D-thiogalactopyranoside

MBP: Mannose binding protein

OGP: Octyl-β-D-glucopyranoside

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

POPC: 1-palmitoyl-2-deoyl-*sn*-glycero-3-phosphocholine

RU: Resonance units

sCD23: Soluble CD23

SCR: Short consensus repeat

sIg: Surface immunoglobulin

SPR: Surface plasmon resonance

SRBC: Sheep red blood cells

TBS: Tris buffered saline

TLCK: L-1-chloro-3-[tosylamido]-7-amino-2-heptanone

CHAPTER 1: INTRODUCTION

1.1. Introduction to CD23

1.1.1. IgE and IgE system

Immunoglobulin E (IgE) is one of the five classes of immunoglobulins in humans (the others being IgM, IgD, IgG and IgA) and it is generally known for its principal role in mediating allergic disorders such as asthma, rhinitis (hay fever), atopic dermatitis, food allergy and anaphylaxis. Allergy is certainly one of the major environmental diseases of modern time and considerable efforts have been made over the past two decades towards understanding the molecular mechanisms involved in allergic reactions. The allergic reaction generally requires three components: allergen, specific antibody and the effector cell. The allergens are multivalent antigens present in our environment in numerous and very different forms, while the antibody is IgE. The third component of the allergic reaction is the effector cell such as mast or basophil cell, which is capable of binding IgE *via* its high affinity receptor, FcεRI. The binding of the allergen to the IgE/receptor complex on the surface of these cells activates a signal transduction mechanism leading to the degranulation of the cell and release of histamine and a number of lymphokines, including interleukin-4 (IL-4), (Paul, 1991). IL-4 activates IgE production *via* a complex regulatory mechanism that culminates in immunoglobulin class switching from IgM to IgE (Finkelman *et al.*, 1990; de Vries *et al.*, 1991) and requires contact between B cell and CD4+ T cell through the interaction of CD40 and CD40L (CD40-ligand), (Armitage *et al.*, 1992).

The newly synthesised IgE binds to its high affinity receptor on mast cells enabling them to bind more allergen which in turn, stimulates them to release more histamine and IL-4, thus creating a local feedback loop. The switched B cells would normally produce predominantly nonspecific IgE capable of binding to mast cells but not able to initiate

their activation; therefore, a threshold level of allergen specific B cells is required for the synthesis of allergen-specific IgE for the repeated contact with the allergen, to maintain the feedback loop (Scharenberg and Kinet, 1994).

The recruitment and activation of the inflammatory cells by the products of degranulation of mast cells induces the expression of the low affinity receptor for IgE (CD23, FcεRII) which is responsible for IgE-dependent cytotoxic activities of the inflammatory cells, such as phagocytosis of the immune complexes by monocytes (Kikutani and Kishimoto, 1990). Apart from this activity CD23 facilitates a number of other IgE dependent or independent activities through the interaction with its other ligands, CR2, CR3 and CR4 (complement receptors type 2,3 and 4). The number of proteins involved in IgE system or activated by its components extends further to two soluble receptor molecules called IgE binding protein (εBP) and IgE-binding factor(s) (IgE-BF). It is clear that although IgE is expressed at very low concentration in human serum (50-300 ng/ml, as compared by 10 mg/ml for IgG), its action is powerfully amplified by the activities of the receptors and other proteins to which it binds, and subsequently by the activities of the proteins activated by these interactions. This has led Sutton and Gould, (1993), to introduce the term "IgE network" comprising IgE and its receptors and CR2 as the second ligand for CD23. The schematic representation of the IgE network and its interaction with complement system through CD23 is given in Fig.1-1.

1.1.2. The structure of IgE

From the structural point of view IgE assembles similarity to the other immunoglobulin classes, comprising two heavy and two light chains, but the IgE ε heavy chain, like the μ chain of IgM consists of five domains, as compared to the four domains in other immunoglobulins. The additional domain (Cε2) is placed into the constant region of the heavy chain in place of the hinge region in IgG and IgA. Unlike IgG, IgE is heat labile and its half-life in the serum is two and a half days, as compared with 21 days for IgG. The restricted proteolysis of IgE generates an Fc fragment (Cε2-Cε4) that still retains the capacity to bind both IgE receptors.

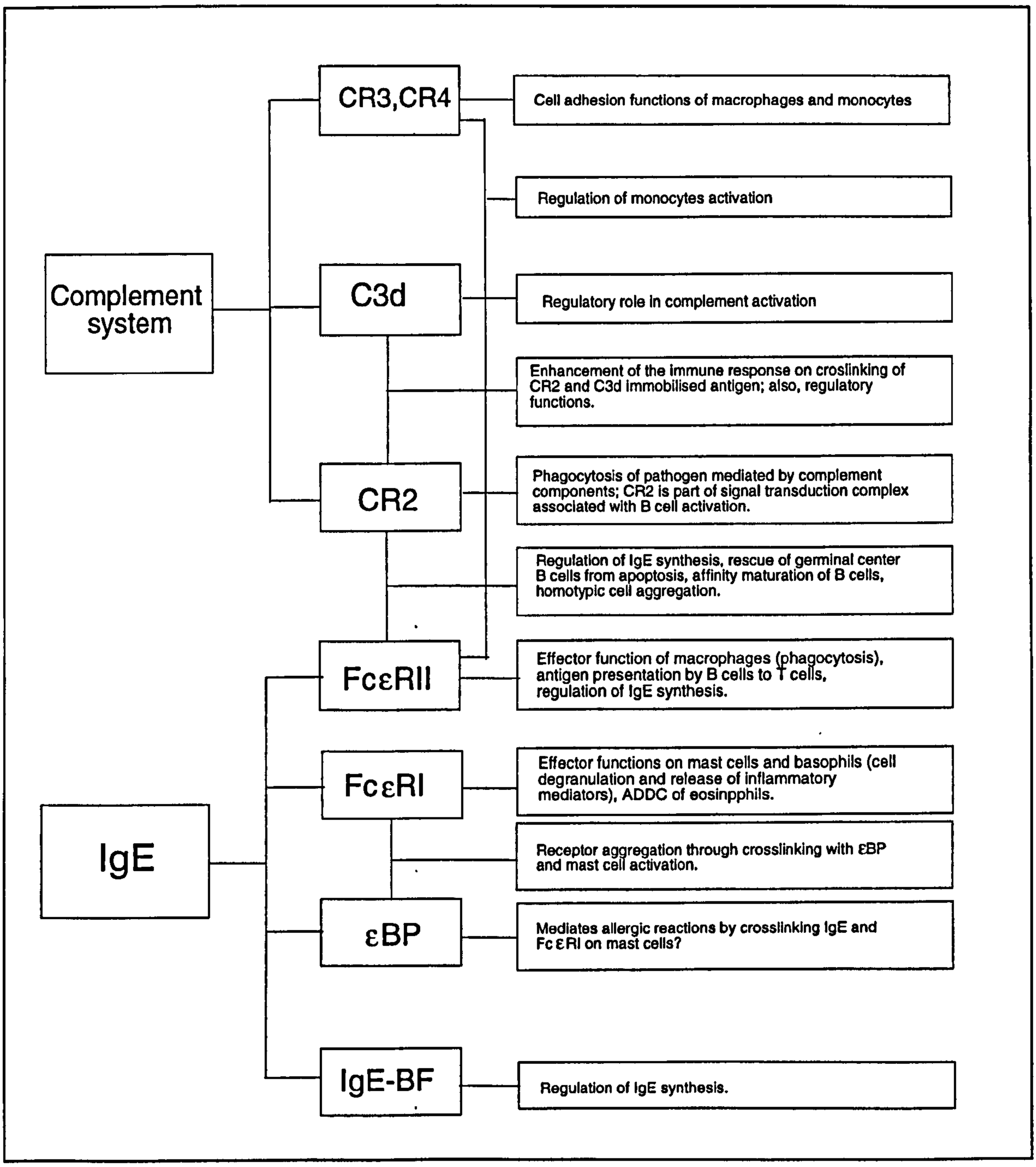


Fig.1-1 The human IgE network and the interaction with complement system.
This schematic representation indicates the major functional consequences of each particular interaction within the human IgE network and focuses on FcεRII/CD23 as the link molecule with complement system. CD23 reacts with three complement receptors (2,3 and 4).

The binding site for FcεRI on human IgE has been initially located to the sequence of 76 amino acids at the Cε2/Cε3 junction (Helm *et al.*, 1988) but it has been now further specified and it has been reported that the first 12 amino acid residues of the Cε3 domain are necessary for binding, with possible contribution of other residues in the domains Cε3 and Cε4 (Beavil *et al.*, 1993; Presta *et al.*, 1993). The binding site for CD23 has been mapped to the same domain (Cε3) but it is distinct from the FcεRI site and it appears to include the amino acids in the region of the glycosylation site Asn 371 (Vercelli *et al.*, 1989b; Ghadery and Stanworth, 1993). Neither of the two IgE receptors requires the carbohydrate moiety of IgE for binding, but the IgE-binding protein (εBP) was shown to bind to IgE in an S-lectin type manner, with the monosaccharide N-acetyllactosamine exhibiting the highest potency in inhibiting the interaction (Hsu *et al.*, 1992).

1.1.3. FcεRI, the high affinity receptor for IgE

The high affinity receptor for IgE (FcεRI) is expressed on the surface of mast and basophilic cells where it initiates allergic reactions (Metzger *et al.*. 1986), but it has been recently found on a number of other cell types including Langerhans cells, eosinophils, monocytes and platelets. Sensitation of mast cells or basophils upon binding of IgE secreted by B cells to FcεRI and crosslinking of the receptors by multivalent antigen induces immediate cellular events such as cellular degranulation and the release of histamine, and synthesis and secretion of a number of cytokines.

FcεRI consists of four transmembrane polypeptides with the composition $\alpha\beta\gamma_2$. The binding site for IgE is in the extracellular part of α chain, while β and γ chains play a role in the insertion of the α chain into the membrane and in signal transduction mediated by FcεRI. The α chain of FcεRI contains two domains characteristic of the immunoglobulin superfamily and IgE binds to the α2 domain (Robertson, 1993). Both domains are glycosylated and the role of the carbohydrates is probably protection from the serum protease activity and receptor expression on the cell surface. These domains each contain a pair of cysteines which most likely form the conserved disulphide bridge. The β chain of FcεRI crosses the plasma membrane four times and has amino and carboxy termini protruding into the cytoplasm while the γ chain has a single transmembrane domain. The

affinity of the assembled receptor for monomeric IgE is about 10^{10} M^{-1} (Miller *et al.*, 1989) and the stoichiometry of one receptor molecule per one IgE molecule was determined by hydrodynamic studies of a complex between the Fc fragment of IgE and a soluble fragment of the FcεRI α domain (Keown *et al.*, 1995).

The mechanism by which FcεRI is activated upon binding the allergen-specific IgE and immobilisation of the allergen still remains somewhat obscure. The aggregation of the receptor molecules on the membrane of the effector cell has been long recognised as an important event in this process (Metzger *et al.*, 1986), but it is unclear what the minimum size of the aggregate is and in particular, what adjacent molecules may be involved. Aggregation of the receptor induces the phosphorylation of non-receptor kinases and other enzymes and the recent studies of the crosslinked IgE/receptor complexes in rat basophilic leukaemia (RBL) cells have revealed that at least two known kinases are associated with FcεRI: the *src*-like kinase p/53/56^{lyn} and the δ isoform of protein kinase C (Mao *et al.*, 1995). Apart from protein kinases this signal transduction pathway involves calcium mobilisation, GTP binding proteins and phospholipid hydrolysis, but there is still no clear evidence about how the aggregation of FcεRI and the influx of the intracellular calcium relate to each other. Several possible pathways for the signal transduction mediated by FcεRI leading to degranulation of the effector cell have been proposed (Ravetch and Kinet,1991).

1.1.4. CD23/FcεRII, the low affinity receptor for IgE: cell type expression

The first demonstration of a cell type other than the mast cell or basophil having receptor for IgE was presented by Lawrence *et al.*, 1975, when it was discovered that human peripheral blood lymphocytes bind myeloma IgE. Following this discovery the second receptor for IgE was found on a variety of haematopoietic cell types, at least in humans. These include macrophages (Capron *et al.*, 1977), eosinophils (Capron *et al.*, 1981), platelets (Joseph, *et al.*, 1983), Langerhans cells (Bieber *et al.*, 1989), T lymphocytes (Yodoi *et al.*, 1979), peripheral monocytes (Melewicz and Spiegelberg, 1980) and also some epithelial cells (Billaud *et al.*, 1989). The application of specific monoclonal antibodies for detection of CD23 has allowed a more detailed re-evaluation of the

expression of this receptor on B lymphocytes and Kikutani *et al.*, 1986b, established that CD23 is expressed on more than 90 % of the human B lymphocytes. Although CD23 expression is restricted to the later stages of B cell development, the CD23 gene is being actively transcribed during all stages, at least in mice (Hagen et al., 1995). This recent finding suggested the existence of a regulatory mechanism for CD23 expression that has not been described yet. As for the T lymphocytes, the presence of CD23 remains somewhat controversial although the evidence for a low-level expression seems to be prevailing. Recent evidence for the expression of allergen-induced CD23 on $CD4^+$ T lymphocytes in patients with allergic asthma may provide clue to resolve this question (Gagro and Rabatic, 1994).

1.1.5. Structure of CD23

In contrast to all other Fc receptors CD23 does not belong to the immunoglobulin superfamily. The C-terminus of the protein is exposed on the cell surface while the N-terminus remains in the cytoplasm, in further contrast to most other cell surface antigens (Kikutani *et al.*, 1986a; Ikuta *et al.*, 1987). The human CD23 molecule is a type 2 integral membrane glycoprotein of about 45 kDa, including one N-linked oligosaccharide at Asn 64 and one or more O-linked carbohydrates (Peterson and Conrad, 1985; Letellier *et al.*,1988; Gould *et al.*, 1991), while murine counterpart is a 49 kDa sialoglycoprotein with two potential N-glycosylation sites (Conrad and Peterson, 1984). Analysis of the genomic sequence of human and murine CD23 shows that the homology between the two proteins is 57 % with a further 17 % of conservative substitutions. Human and murine CD23 both have discrete structural domains, as shown on the model proposed by Gould et al., 1991, in Fig.1-2.

The human CD23 exists in two forms (termed *a* and *b* in Fig.1-2) differing only in the N-terminal 6/7 amino acid residues (Yokota *et al.*, 1988). The distinct domains of CD23 are the C-terminal ligand binding domain (known as, and referred to in the following text, "lectin" domain), the "stalk" region which links the lectin domain with the membrane, the hydrophobic transmembrane region and the short cytoplasmic N-terminal sequence. C-terminal to the lectin domain there is a short sequence containing a reverse RGD motif

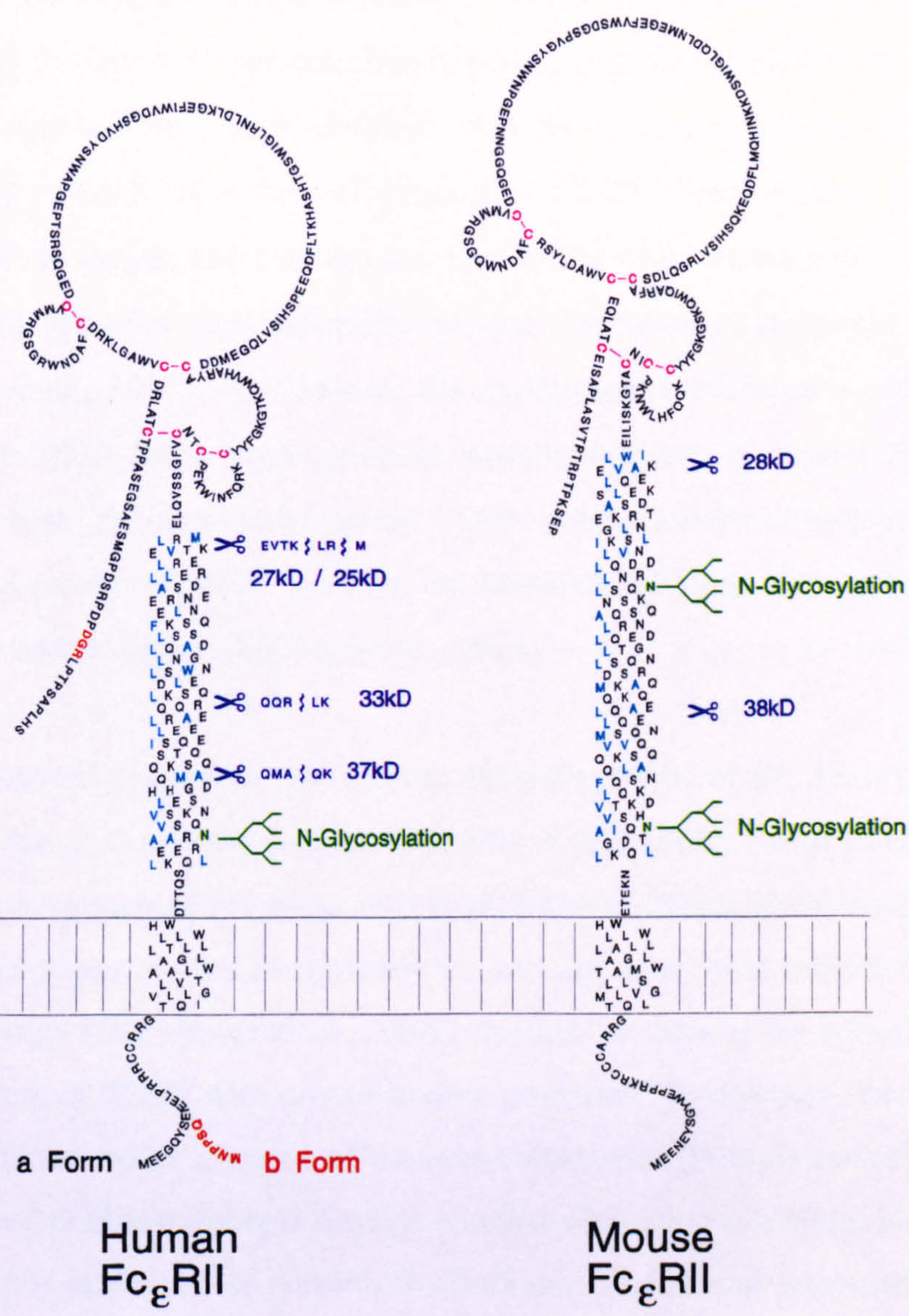


Fig.1-2 The structure of human and murine CD23 (Gould *et al.*, 1991).
The model shows the distinct structural domains of CD23; the lectin homology domain is linked to the cell membrane by a sequence that is predicted to form triple α-helical structure and is referred in the text as the stalk region; the (auto)proteolytic cleavage sites and the single N-glycosylation site in human CD23 or two sites in mouse protein are shown. The human CD23 exists in two forms, *a* and *b*, the difference between the two being only in the short N-terminal sequence, as indicated on the model. The further difference in the structure between human and mouse CD23 is reflected in the extracellular C-terminal sequence that flanks the lectin domain; in human CD23 this sequence contains a reverse integrin binding tripeptide (DGR) which is not present in mouse CD23.

that may act in cell adhesion. The homology of the lectin domain of CD23 to asialoglycoprotein receptor and also a number of adhesion proteins known as selectins places CD23 in the family of C-type (calcium dependent) lectins (Drickamer, 1988). The lectin domain is flanked on the N-terminal side by a sequence containing heptad hydrophobic repeats which form the stalk region of CD23. These repeats are each 21 amino acid residues in length, and they are arranged in the way that resembles the leucine zipper motif which provides the mechanism for the formation of α-helical coiled coil structure (Beavil *et al.*, 1992). And indeed, the experimental evidence for this type of oligomerisation of CD23 came from chemical crosslinking data of Beavil *et al.*, 1995, who showed that both the membrane bound CD23 and its soluble fragments can form protein dimers and trimers. Similar work on the murine CD23 has shown that it is also oligomeric and at least a trimer (Dierks, *et al.*, 1993).

CD23 undergoes natural proteolytic cleavage to yield fragments of 37, 33, 29, 25 and 16 kDa all of which retain the lectin domain (Letellier *et al.*, 1989). There is considerable controversy about the nature of the protease(s) that cleave(s) CD23. There are suggestions that CD23 itself may possess the autoproteolytic activity or at least require the 37 kDa fragment for cleavage (Delespesse et al., 1992). However, there is no homology in the amino acid sequence of CD23 with any of known proteases. Two reports have appeared recently pointing at the major allergen of the house dust mite (Der pI) as the proteolytic source that cleaves CD23 and disrupts the IgE network (Schulz *et al.*, 1994; Hewitt *et al.*, 1995). Two protease inhibitors of proteolytic cleavage, TLCK and iodoacetamide, can inhibit proteolytic degradation of CD23 (Cairns and Gordon, 1990).

1.1.6. Biological function of CD23

CD23 participates in several cellular events and its functions can be generally divided into two categories: IgE-dependent and IgE-independent.

a) IgE dependent activities of CD23

The involvement of CD23 in IgE biology was first demonstrated by Capron *et al.*, 1975,

who showed that CD23 participates in IgE-dependent cytotoxic activities of inflammatory cells such as phagocytosis of antigen complexes by macrophages. Subsequently, the similar role for CD23 was described in eosinophils (Capron *et al.*, 1981), platelets (Joseph *et al.*, 1983) and monocytes (Kikutani and Kishimoto, 1990). In each of these cells CD23 binds IgE/antigen complexes and facilitates their phagocytosis.

The role of CD23 in IgE-mediated antigen presentation has become the focus of a number of recent studies, *in vitro* and *in vivo*, as well. Analogous to Fcγ receptors, CD23 acts synergistically with other proteins, namely with the complement receptors CD11b and CD11c, the α chains of the β_2 integrin adhesion molecule complexes CD11b-CD18 and CD11c-CD18 (Capron *et al.*, 1987; Lecoanet-Henchoz *et al.*, 1995). Biochemically, once bound to CD23 the IgE/antigen complex can be internalized by receptor mediated endocytosis, followed by proteolysis of the receptor, IgE and the antigen, and the re-expression of the antigen fragments on the cell surface, making them accessible for the interaction with T cells (Chen, 1991). Using hapten specific IgE, Kehry and Yamashita, 1989, showed in mice that antigen presentation by B cells to an antigen-specific T cell line could be enhanced up to 100-fold as compared to the antigen alone or with targeting through FcγRII receptor (the low affinity receptor for IgG) using IgG1. These data have been recently extended in the human system by demonstration that human EBV-transformed B cells can internalize and present IgE/antigen complexes *via* CD23, thus confirming the general nature of the phenomenon (Pirron *et al.*, 1990). In 1994, Fujiwara *et al.* published the data on CD23-deficient mice demonstrating that although the other known functions of CD23 may exist *in vivo*, the IgE-mediated antigen presentation was the only one unique for CD23, at least in mice. The possible consequence of the CD23/IgE-mediated antigen presentation in allergy is a continuous activation of the immune system by very low concentrations of allergen, which could lead from sensitivity to a single group of allergens into sensitivity to multiple groups of allergens (Mudde *et al.*, 1995).

In addition to IgE-mediated antigen presentation and cytotoxic clearance of the antigen, CD23 is reported to participate in the control of IgE synthesis, a role which has been suggested on the basis of several lines of evidence. In general, the regulation of IgE

synthesis by membrane CD23 and its soluble fragments is dependent on the stage of B cell development and can be either positive or negative (Sarfati *et al.*, 1988, 1992a, 1992b; Sutton and Gould, 1993). The stimulative role of soluble CD23 (sCD23) in modulating IgE synthesis is IL-4 dependent and characteristic of IgE committed B cells (Saxon *et al.*, 1990). This is supported by the observation that some anti-CD23 monoclonal antibodies inhibit the Il-4 stimulated IgE production in human peripheral blood or by tonsillar lymphocytes (Pene *et al.*, 1988; Bonnefoy *et al.*, 1994), presumably by neutralizing the IgE-potentiating activity of sCD23 or preventing the proteolytic cleavage of membrane CD23. The negative regulation of IgE synthesis by membrane CD23 requires the binding of IgE itself to the membrane CD23 and possibly also crosslinking of the membrane CD23 and IgE. Anti-IgE monoclonal antibodies can inhibit binding of IgE to the membrane CD23 and thus stimulate IgE synthesis *in vitro* (Sherr *et al.*, 1989). The negative regulatory role of membrane CD23 in the production of IgE was confirmed *in vivo* by the demonstration that CD23 deficient mice showed an increased and sustained specific IgE antibody titre (Yu *et al.*, 1994), Therefore, the IgE suppressive activity of CD23 through interaction with IgE may be viewed as a negative feedback loop in the IgE synthesis (Sutton and Gould, 1993).

b) IgE-independent functions of CD23

The first indication that FcεRII is the same molecule as the B cell differentiation antigen CD23 came in 1987 (Bonnefoy *et al.*; Yukawa *et al.*) suggesting that this protein may be involved in the biology of the B cell independent of its role as the IgE receptor. MHM6, an antibody to CD23, provides a progression signal to human B cells primed to enter the G1 phase of the cell cycle by phorbol ester and also stimulates IL-4 promoted DNA synthesis (Gordon, 1994). In contrast to that, some other anti-CD23 antibodies and IgE/antigen complexes have been found to down-regulate Il-4-dependent stimulation of B cells (Delespesse et al., 1992). The signal transduction pathway activated by CD23 involves a rapid increase in intracellular calcium as the consequence of generation of inositol-triphosphate, following the phospholipase C catalyzed hydrolysis of phosphatidylinositol-biphosphate (Kolb et al., 1991; Kolb and Abadie, 1992). At present it is not clear what initiates this cascade of events but a tyrosine kinase seems to be

involved, the evidence for which was provided by a study demonstrating an association between CD23 (isoform *a*) and the *src* related protein kinase $p59^{fyn}$ (Sugie et al., 1991). The activation of phospholipase C by phosphorylation triggers adenyl-cyclase activation and leads to accumulation of cAMP, although the generation of cAMP can be triggered independently from mobilisation of Ca^{2+} ions (Kolb et al., 1993). It is interesting that the two isoforms of CD23 (*a* and *b*) appear to facilitate different signal transduction pathways, and this fact must be attributed to the difference in the first 6/7 amino acids of their cytoplasmic N-termini (Gordon, 1994). Both isoforms trigger accumulation of cAMP, but only the isoform *a* is coupled to phospholipase C hydrolysis of phospatidylinositol-biphosphate.

The structure of CD23 may have important implications for the signal transduction mediated by this protein. Gould et al., (1991) have suggested that a conformational change in the structure of CD23 upon binding IgE or anti-CD23 monoclonal antibodies may play an important role; IgE binding stabilizes the α-helical coiled coil structure of the stalk region and thus prevents proteolytic cleavage of CD23; the binding of a monoclonal antibody produces an opposite effect and results in the cleavage of the protein.

Several studies have focused on the potential function of sCD23 as an autocrine B cell growth factor (Mossalayi *et al.*, 1991; Gordon *et al.*, 1985) but this appears to be the most controversial function of CD23 of all. Some of the controversy can be attributed to the wide heterogeneity of soluble CD23 fragments. Delespesse *et al.*, 1989, showed that a 12 kDa CD23 fragment promotes the proliferation of B cells whereas Gordon *et al.*, 1989, reported that 37 kDa fragment is responsible for this function. Further to that, Armitage and Goff, 1988, reported that stimulation of cell growth by sCD23 is conditional and requires the interaction between cells. The availability of recombinant soluble forms of CD23 should provide the necessary means to study the possible role of CD23 in stimulating B cell growth. And finally, it has been proposed that sCD23 in synergy with IL-1 is involved in the rescue of the centrocytes from programmed cell death by apoptosis (Liu *et al.*, 1991). This finding is supported by the evidence of expression of CD23 on follicular dendritic cells (FDC) located at the same place as the centrocytes. Bonnefoy *et al.*, 1993, presented the evidence that the involvement of CD23 in the rescue of germinal

center cells from apoptosis is exerted through its interaction with CR2. The interaction of CD23 and CR2 and its possible functional implications will be dealt with in more detail later on in this thesis. A summary of biological functions of CD23 is given in Table 1-1.

1.1.7. Lectin properties of CD23

As mentioned before, CD23 belongs to the family of C-type animal lectins which also includes asialoglycoprotein receptor, chicken hepatic lectin, mannose binding protein, tetranectin, Lyb-2 B cell antigen and other proteins. All of these proteins share a common sequence consisting of 14 amino acid residues, which acts as the carbohydrate binding domain (Drickamer and Taylor, 1993). The highly conserved residues include 4 cysteines that form two disulphide bonds and also glutamic acid, aspartic acid and asparagine, while several glycine and proline residues are found at the critical turn positions in the structure. There also appears to be a subfamily of structurally related C-type lectins comprising α-helical coiled coil stalks of different lengths, of which CD23 is a member (Beavil *et al.*, 1992).

Although IgE binds to the lectin domain of CD23 and requires Ca^{2+} for binding, the interaction is not mediated by the carbohydrate chains of IgE, as shown by the demonstration of the ability of IgE ε-chain fragments synthesized in *E. coli* to bind CD23 (Vercelli *et al.*, 1989b). The first demonstration of lectin function of CD23 came in 1992 when Pochon et al. reported that CD23 binds to a ligand different from IgE and this second ligand was found to be CR2 (CD21), the complement receptor for C3dg fragment of the complement (Aubry et al., 1992). The binding was found to be Ca^{2+} dependent and the tunicamycin treatment of the CR2 expressing cells significantly decreased binding of CD23. Also, the authors reported that the monosaccharide fucose-1-phosphate inhibited the binding of CD23 to its ligand suggesting the involvement of a glycan structure containing, or related to, fucose-1-phosphate type. This was further reinforced by the finding that the glycosylation mutants of CR2 failed to bind CD23 and that 2 N-linked oligosaccharides are involved in interaction (Aubry *et al.*, 1994). However, another line of evidence implicated the monosaccharide galactose as the principal carbohydrate component of glycoproteins involved in interaction with CD23 (Kijimoto-Ochiai *et al.*,

Table 1-1: Summary of biological functions of CD23 (Some of the representative references for each function are given).

IgE dependence	Biological function	Reference
IgE-dependent	Cytotoxic clearance of antigen	Capron *et al.*,1975,1981; Joseph *et al.*,1983;
	Antigen presentation	Capron *et al.*,1987; Kehry and Yamashita, 1989; Pirron *et al.*, 1990; Chen, 1991; Fujiwara *et al.*, 1994, Gordon *et al.*, 1991
	Control of IgE synthesis	Pene *et al.*,1988; Saxon *et al.*,1990; Sarfati *et al.*, 1992, Sutton and Gould, 1993; Bonnefoy *et al.*, 1994; Yu *et al.*, 1994
IgE-independent	B cell activation	Gordon, 1983, 1989; Delespesse *et al.*, 1992
	Autocrine growth factor	Gordon *et al.*, 1985, 1991; Delespesse *et al.*, 1989; Mossalayi *et al.*,1991
	Rescue of germinal center B cells from apoptosis	Liu *et al.*, 1991; Bonnefoy *et al.*, 1993
	Adhesion protein	Bjorck *et al.*, 1993; Lecoanet-Henchoz *et al.*,1995
	Other functions: prothymocyte maturation, myeloid precursor proliferation, inhibition of macrophage migration	Mossalayi *et al.*, 1990b, 1990a; Flores-Romo *et al.*, 1989

1994). The report further indicated that CD23 binds with higher affinity the structure Gal-GalNAc (O-type sugar) than Gal-GlcNAc (N-type sugar). The same authors (Kijimoto-Ochiai and Uede, 1995) then showed that CD23 acts as a galactose-binding lectin in the cell aggregation of EBV-transformed human B cells. They showed that naturally aggregated $CD23^+$ EBV-transformed cells disaggregated after removal of galactose by β-galactosidase treatment or by addition of anti-CD23 monoclonal antibodies, as well as soluble CD23. Interestingly, anti-CR2 antibodies failed to exhibit the same effect and the authors suggested that CD23 may react with a ligand other than CR2.

Overall, these reports showed that CD23, apart from binding IgE does indeed function as a lectin protein but the evidence for its carbohydrate specificity is still insufficient and apparently controversial. Clearly, more work is required to establish the carbohydrate binding specificity of CD23.

1.1.8. IgE-binding protein (εBP)

The existence of εBP was revealed when Liu *et al.*, 1989, sequenced a cDNA clone encoding for a 31 kDa protein which was regularly copurified with the high affinity receptor for IgE from the rat basophilic leukaemia (RBL) cells, and was actually thought to be one of the subunits of FcεRI. The biological function of the protein was not known for some time until it was established that the carbohydrate-binding protein (CBP35) was the mouse counterpart of εBP, which has led to the classification of εBP as an endogenous S-type lectin. Soon after, it was also discovered that this protein had been long known in humans as Mac-2 (a macrophage differentiation marker) and L-29 (a lectin initially identified in human lungs).

The tissue distribution of εBP is widespread and the specificity for β-galactoside containing oligosaccharides is not uncommon, thus making it unlikely that εBP is specific only for IgE. Multiple functions have been proposed for this protein but to this day no clear evidence about its role in either IgE biology or in general, have been presented. The lack of clarity could be explained by the mentioned broad specificity and tissue distribution, in addition to the fact that different researchers still use different names for

this protein which inevitably adds to the difficulty in searching for a function unique to εBP. Nevertheless, several suggestions about the function of εBP have been made, based on both, experimental and hypothetical evidence. IgE binding activity of εBP points to a possible role in IgE mediated allergic reactions (Robertson *et al.*, 1990); CBP35 has been implicated in regulation of cell growth (Laing and Wang, 1988); the studies with Mac-2 suggested that this protein may be a differentiation marker of macrophages (Cherayl *et al.*, 1989); the finding that the laminin binding protein L-29 is identical to CBP35/Mac-2/εBP suggests a possible role in cell adhesion to the extracellular matrix (Woo *et al.*, 1990).

Like many other lectins, εBP is bivalent and the studies with the recombinant protein expressed in *E. coli* showed that εBP possesses the ability to self-associate (Hsu *et al.*, 1992). Massa *et al.*, 1993, have shown that L-29 binds to glycoconjugate ligands such as laminin with a positive cooperativity between the protein subunits. Together with the discovery that εBP binds not only IgE but also FcεRI (Frigeri *et al.*, 1993), this finding reinforces a possible role for εBP in allergic reactions. A model has been suggested in which εBP activates mast cell by crosslinking the IgE receptors, receptor-bound IgE or IgE and the receptor, and thus provides the mechanism for receptor aggregation leading to mast cell activation (Liu, 1993). The function of εBP as a modifier of cellular response may then be extended to other cell types expressing glycoprotein ligands for this lectin.

1.1.9. IgE binding factors

The term "IgE binding factors" (IgE-BFs) was originally used for the low molecular weight soluble proteins that were able to bind IgE and potentiate IgE synthesis (Sarfati *et al.*, 1984; Metzger, 1988). With the studies of molecular structure of CD23 it became clear that IgE-BFs are in fact proteolytic cleavage products of CD23, namely 25 and 16 kDa fragments. However, in rats there appear to exist two IgE-BFs not related to CD23. Both factors bind IgE but have contrasting effects on IgE synthesis: IgE-PF potentiates while IgE-SF suppresses IgE synthesis (Ishizaka, 1988). Cloning of cDNA for these proteins revealed that they are encoded by a single gene (Martens *et al.*, 1987) and the only difference between them was the presence of N-glycans on IgE-PF (Ishizaka, 1988).

Other studies showed that a single T cell can produce either IgE-PF or IgE-SF, depending upon the presence of a regulatory protein GIF (glycosylation inhibitory factor) which can down-regulate the production of IgE-PF (Uede *et al.*, 1983). The IgE-PF is 60 kDa glycoprotein which can be cleaved to produce a 11 kDa fragment that still retains the ability to potentiate IgE synthesis (Martens *et al.*, 1985).

Most of these studies have been performed with rat T lymphocytes and very little is known about human IgE-BFs. The critical issue with these molecules is establishing better their role in the synthesis of IgE and examining the possible clinical implications in suppressing IgE mediated allergic reactions.

1.2. Introduction to CR2 (CD21)

1.2.1. Complement system

The complement system has evolved as one of the major effector pathways in the process of inflammation. It is composed of a number of distinct plasma proteins which are termed C1, C2,...C9 but the actual sequence of reactions is: C1-C4-C2-C3-C5-C6-C7-C8-C9. The activation of the complement occurs when one of the complement proteins binds antibody and triggers a cascade of reactions activating all other components. The activation of the complement system by an antibody binding to antigen is part of the adaptive humoral immune response but there is also an alternative pathway by which the complement system takes part in the early induced (innate) response to the pathogen.

Once the complement system is activated it leads to proteolytic cleavage of the complement components and one of the major events in the first phase of complement activation is the cleavage of C3 to produce C3b which binds covalently to the surface of the pathogen. The marking of the pathogen with C3b exposes it to the phagocytic cells that can bind C3b through complement receptors (CRs) expressed on these cells. C3b also activates the alternative pathway, which deposits more C3b on the surface of the pathogen

leading to opsonization of the pathogen. The terminal components of the complement (C5-C9) polymerize to form a membrane attack complex which can inflict pores in the membrane of the pathogen and in that way destroy it.

The formation of C3b fragment is catalyzed by the enzyme called C3/C5 convertase which is activated by the cascade of the following reactions (Janeway and Travers, 1994): The first component of complement (C1) is composed of 3 proteins, C1q, C1r and C1s, in such a way that each C1q molecule has attached to it two molecules of C1r and C1s. Binding of the antibody attached to the surface of pathogen occurs through C1q, which requires at least two antibody molecules for activation. Soluble antibodies free of antigen cannot bind to C1q because of inaccessibility of the binding site. The activation of C1q is followed by the enzymatic activity of C1r which cleaves C1s to generate an active serine protease, which in turn cleaves C4 to produce C4b, and also cleaves C2 to produce C2b, which is itself a serine protease. C2b is generally called C3/C5 convertase and its generation from the C2 component takes place on the surface of the pathogen, to which it is bound *via* C4b. The main product of C3/C5 convertase activity is C3b which, as mentioned before, binds in large numbers to the surface of the pathogen, but also important is the C3a fragment which initiates a local inflammatory response. Finally, C3b binds C5 leading to the generation of C5b that associates with the pathogen membrane and triggers the polymerisation of the terminal components of the complement and formation of the membrane-attack complex capable of damaging the membrane of the pathogen. The alternative pathway of complement activation can amplify the effect of this classical pathway by depositing more molecules of C3b which are generated by a positive loop comprising factor B which binds to C3b and becomes cleaved by factor D, resulting in the formation of the C3b-Bb complex that possesses C3/C5 convertase activity. Thus, new C3b is generated and at this point the two pathways converge. A list of the complement components, their active fragments and related functions in the order of activation is shown in Table 1-2.

Table 1-2: The proteins of complement system and their functions, in the order of activation

Native component	Active form	Function
C1	C1q	Binds to antibody/antigen complex on pathogen. Activates C1r.
	C1r	Cleaves C1r to generate protease C1s.
	C1s	Cleaves C4 and C2.
C4	C4a	Mediator of inflammation.
	C4b	Covalently binds to pathogen and opsonizes it. Binds C2 for cleavage with C1s.
C2	C2a	Unknown
	C2b	C3/C5 convertase (cleaves C3 and C5).
C3	C3a	Mediator of inflammation.
	C3b	Binds to pathogen in large numbers and opsonizes it. Initiates positive loop of alternative pathway. Binds C5 for cleavage with C2b.
C5	C5a	Mediator of inflammation.
	C5b	Initiates formation of membrane-attack complex.
C6	C6	Binds C5b and accepts C7.
C7	C7	Binds C5b,C6. Complex insertion into membrane.
C8	C8	Binds complex C5b/C6/C7. Initiates C9 polymerisation.
C9	C9	Polymerises to C5b/C6/C7/C8 to form a channel in the membrane.

1.2.2. Complement receptors

The actual destruction of pathogen is facilitated by phagocytic cells which are abundant in complement receptors and therefore can bind the complement marked pathogen. There are five known complement receptors: CR1(CD35), CR2 (CD21), CR3 (CD11b-CD18), CR4 (CD11c-CD18) and C1q receptor. CR1 is the best known complement receptor and is expressed on both macrophages and polymorphonuclear leucocytes. CR1 binds C3b component of C3 (Gigli and Nelson, 1968) but it is not sufficient alone to trigger phagocytosis in a macrophage. It also serves as a cofactor for the serine esterase Factor I which cleaves C3b to iC3b and C3f, and subsequently iC3b to C3c and C3dg (Ahearn and Fearon, 1989). All of the cleavage products of C3b are inactive and therefore, CR1 has an important regulatory function. In addition to that, CR1 may serve in conjunction with CR2 as a receptor for mediating lymphocyte activation.

The role of CR2, CR3 and CR4 is the binding of inactivated forms of C3b that remain attached to the pathogen surface. CR2 is capable of binding of all three degradation fragments of C3b (iC3b,C3dg,C3d) (Ross and Cambris, 1982). C3 and C4 belong to a superfamily of structurally related cell surface receptors and adhesion molecules which includes the fibronectin receptor and other integrins. These receptors bind to their ligands in a calcium dependent manner and have a common binding specificity for the tripeptide RGD (Arg-Gly-Asp). The receptor for the C1q component of complement is the least characterised of the complement receptors. Table 1-3 shows the binding specificities of the complement receptors and cell types expressing them.

1.2.3. Expression, structure and function of CR2 (CD21)

The co-expression of CR2 and EBV receptor on multiple B cell lines had been known for some time but it is the studies of Fingeroth *et al.*, 1984, that presented the evidence that CR2 and EBV receptor are the same protein. They showed that modulation of CR2 from the surface of B cells with the specific anti-CR2 antibody HB5 blocked EBV binding. This was later confirmed more directly by showing that CR2 incorporated into liposomes can bind EBV, as well as C3d (Mold *et al.*, 1986).

Table 1-3: The complement receptors (CRs) and their binding specificities

Receptor	CD name	Binding specificity	Cell type expression
CR1	CD35	C3b,C4b	Macrophages, erythrocytes, monocytes, polymorphonuclear leucocytes, B cells
CR2	CD21	C3d,C3dg, iC3b	B cells
CR3	CD11b-CD18	iC3b	Macrophages, monocytes, polymorphonuclear leucocytes
CR4	CD11c-CD18	iC3b	Macrophages, monocytes, polymorphonuclear leucocytes
C1qR	none	C1q	B cells, macrophages, monocytes, platelets, endothelial cells

The expression of CR2 is also characteristic of IgM^+/IgD^+ mature B cells and it is rapidly lost following cellular maturation. Follicular dendritic cells (FDC) in germinal centers are also CR2 positive but the function of CR2 on these cells is not well known. Later, the distribution of CR2 was expanded to include epithelial cells (Sixbey *et al.*, 1983) and T lymphocytes (Fingeroth *et al.*, 1988; Watry *et al.*, 1991).

CR2 is heavily glycosylated 140 kDa protein containing 11 potential glycosylation sites occupied by N-linked but not O-linked oligosaccharides (Weis and Fearon, 1985). The carbohydrate content of CR2 is not required for binding to its complement ligand C3d, as shown by the capacity of unglycosylated or underglycosylated CR2 protein precursor to bind to C3 immobilised to Sepharose (Weis and Fearon, 1985). Several antibodies to CR2 have been generated and the studies using these antibodies as potential blockers in binding to C3d or EBV have indicated that CR2 possesses discrete structural domains with distinct functional properties (Cooper *et al.*, 1988). The entire extracellular domain of CR2 is composed of 60-75 amino acid tandem repeats or short consensus sequences (SCRs) and there are 15 or 16 of these in the structure of CR2, depending on the (non)splicing of the SCR 11 (Fujisaku *et al.*, 1989). The 60-75 amino acids SCR structure of CR2 places it into the group of 20 or more related cell membrane or plasma proteins, some of which are complement related molecules (CR1, C4 binding protein, C2, Factor B, Factor H) and the others non-complement related molecules such as β2-glycoprotein I, β chain of IL-2 receptor, blood clothing factor III, etc. (Cooper *et al.*, 1988). Each SCR has four cysteine residues that are probably disulphide bonded in a cys1-cys3, cys2-cys4 pattern forming a triple loop structure (Janatava *et al.*, 1989). The binding sites for C3d and EBV are contained within the SCRs 1 and 2.

The hydrophobic transmembrane domain of CR2 is composed of 28 amino acid residues and this is followed by a 34 amino acids cytoplasmic sequence that among the other residues comprises 3 serines, 2 threonines and 4 tyrosines, including a protein kinase C motif TSQK. Although the cytoplasmic domain of CR2 is too small to encode a tyrosine kinase it could serve as a substrate for phosphorylation, as can be suggested from the presence of sequence EAREVY, a common motif for tyrosine kinase substrates (Ahearn and Fearon, 1989). The schematic representation of CR2 molecule with indicated binding

sites for different ligands or monoclonal antibodies is shown in Fig.1-3.

Apart from binding C3d and the gp350/220 of EBV, CR2 is also an interferon-α receptor (Delcayre *et al.*, 1991) and also interacts by its intracytoplasmic carboxy-terminal domain and two distinct binding sites with p53 anti-oncoprotein and the p68 calcium-binding protein (Frade *et al.*, 1992), both of which are intracellular regulatory proteins. Carter *et al.*, 1988, have shown that crosslinking of CR2 and IgM on the B cell surface with monoclonal antibodies to each protein produces a synergistic increase in intracellular calcium. CR2 also associates with CD19, CD81 (TAPA-1) and Leu-13 to generate a signal transduction complex that is independent of the antigen receptor (Fearon and Carter, 1995), the role of which is closely associated with B cell activation and development regulation (Tedder *et al.*, 1994). The recent discovery that CR2 binds to the low affinity receptor for IgE, CD23 (Aubry *et al.*, 1992) further strengthens the role of CR2 in B cell development and provides both proteins with the role of an adhesion pair in B-B or B-T cell interactions.

1.3. Interaction of CD23 and CR2

1.3.1. Discovery

The demonstration that CD23 has a second ligand apart from IgE came in 1992 when Pochon *et al.*, showed that CD23 incorporated in liposomes binds to a number of cell types in a specific manner and, of these, the two myeloma B cell lines RPMI 8226 and U266 B1 were shown to be the most positive, while Daudi and Burkitt lymphoma cells, as well as T cells and non-lymphoid cells were negative. The binding of CD23-liposomes to the cells was shown to be calcium dependent with a maximum binding at 2 mM Ca^{2+} and inhibited by the monosaccharide fucose-1-phosphate. Also, tunicamycin (an inhibitor of N-glycosylation of proteins) treatment of the cells greatly reduced binding of CD23-liposomes, suggesting a possible carbohydrate involvement. The binding could be inhibited by a number of anti-CD23 monoclonal antibodies and to some extent, by IgE. Following

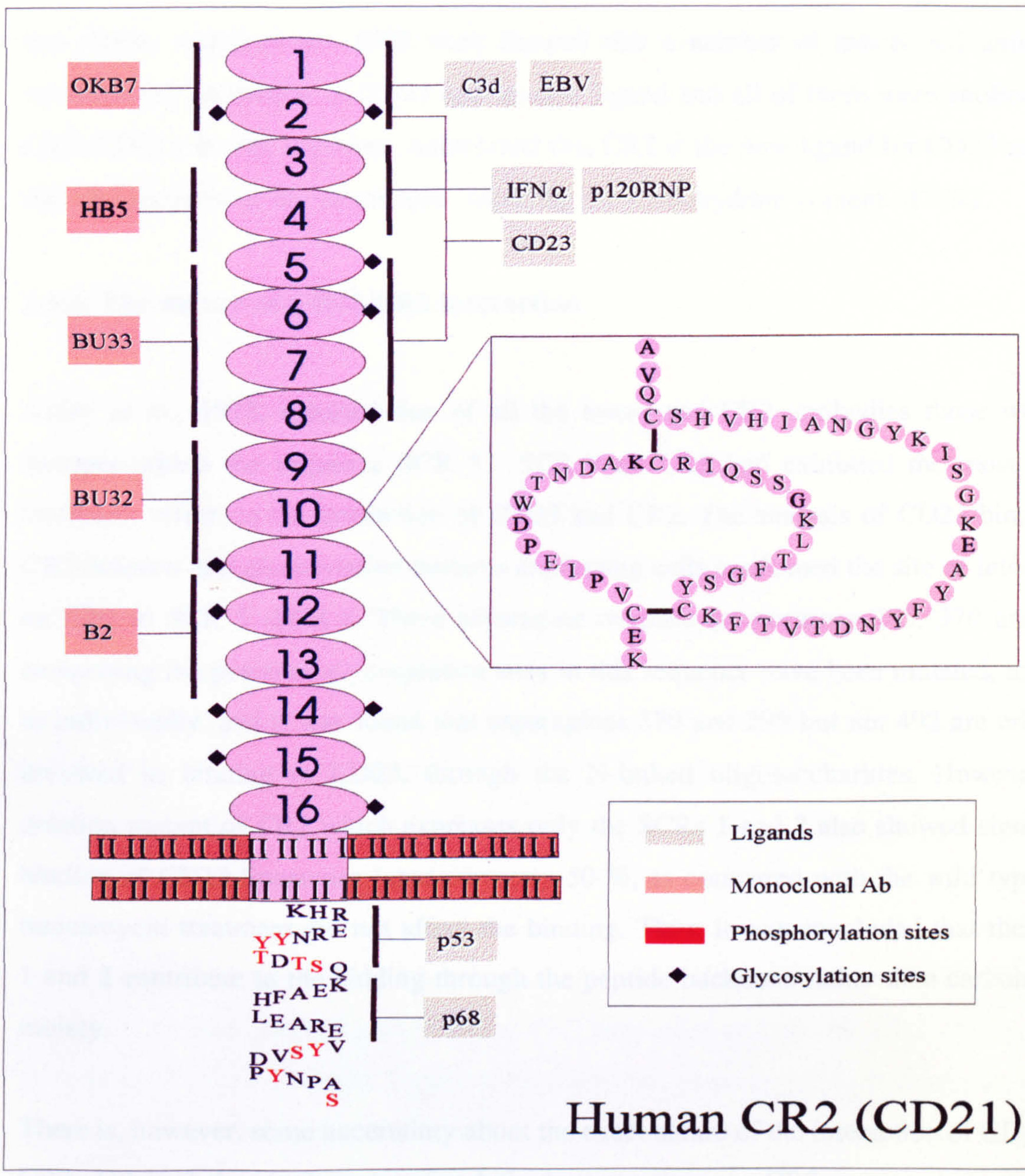


Fig.1-3 The structure of human CR2/CD21.

This model of human CR2 depicts its "beads on a string" like structure and indicates the binding sites of its seven known ligands: C3dg, EBV, CD23, interferon-α (IFN-α), ribonucleoprotein p120, calcium binding protein p68 and anti-oncoprotein p53; it also indicates the binding sites of several, more often used monoclonal antibodies to this protein. The number of SCRs (short consensus repeats) in CR2 can be either 16 or 15, depending on the (non)splicing of SCR 11 in the mRNA transcript; each SCR is thought to contain two S-S bonds that are formed between cysteine residues 1-3 and 2-4; the insert represents a simplified model of one SCR (number 10) by analogy to the structure of SCRs in C4b binding protein (Janatava *et al.*, 1989). Shown in red are the residues that can be phosphorylated, in the cytoplasmic sequence of CR2.

this report, Aubry *et al.*, 1992, then showed that a number of anti-B cell antibodies inhibited the interaction of CD23 and its new ligand and all of them were antibodies to CR2 (CD21). It was, therefore, established that CR2 is the new ligand for CD23 and that the interaction is of the lectin type, requiring the carbohydrate content of CR2.

1.3.2. The nature of CD23-CR2 interaction

Aubry *et al.*, 1992, reported that of all the tested anti-CR2 antibodies those with the epitopes within the sequence SCR 5 - SCR 8 on CR2 had exhibited the most potent inhibitory effect on the interaction of CD23 and CR2. The analysis of CD23 binding to CR2 deletion and glycosylation mutants expressing cells confirmed the site of interaction on CR2 to SCR 5 -SCR 8. Three asparagine residues (at positions 295, 370 and 492) comprising the potential glycosylation sites in this sequence have been mutated, together or individually, and it was found that asparagines 370 and 295 but not 492 are critically involved in binding to CD23, through the N-linked oligosaccharides. However, the deletion mutant of CR2 which expresses only the SCRs 1 and 2 also showed significant binding of CD23-liposomes (approximately 50 %, as compared with the wild type) and tunicamycin treatment did not affect the binding. Thus, it was concluded that the SCRs 1 and 2 contribute to the binding through the peptide backbone rather than carbohydrate moiety.

There is, however, some uncertainty about the exact nature of the interaction of CD23 and CR2. Although the involvement of carbohydrate structures on CR2 seems to be reasonably well documented, there is some inconsistency in evidence for actual contribution of the carbohydrates to the binding. For example, Aubry *et al.*, 1994, reported that tunicamycin treatment of CR2 expressing cells can greatly reduce the binding of CD23-liposomes (to less than 20 % of the positive control), while the deletion mutant comprising only SCRs 1 and 2, which contributes to the binding by its peptide bone, alone retains already 50 % binding. Also, some of the CR2 positive cell types did not bind CD23-liposomes and this was explained by the fact that the glycosylation of CR2 in these cells is not appropriate for binding to CD23 (Aubry *et al.*, 1992, 1994; Pochon *et al.*, 1992); yet, if the peptide sequence of SCRs 1 and 2 contributes to the binding by half, some binding should be

observed in all CR2 positive cell types. And finally, fucose-1 phosphate was shown capable of inhibiting the interaction of CD23 and CR2 (Pochon *et al.*, 1992), while other studies on lectin properties of CD23 indicated galactose as the principal component of the glycoproteins involved in binding to CD23 (Kijimoto-Ochiai *et al.*, 1994; Kijimoto-Ochiai and Uede, 1995). Obviously, more studies are necessary to establish the exact nature of the interaction between CD23 and CR2.

1.3.3. Functional implications

Aubry *et al.*, 1992, showed that interaction of CD23 and CR2 is not merely physical but has a regulatory function in IgE synthesis. They showed that engagement of CR2 by anti-CR2 antibody BU-33 or soluble CD23 increases IgE production. A possible explanation of this effect could be that the interaction of CD23 and CR2 provides an enhancement of the T cell-B cell contact which is required for IgE production (Vercelli *et al.*, 1989a), and the particular characteristic of the CD23-CR2 interaction is that this pairing controls IgE synthesis in an isotype specific manner (Bonnefoy *et al.*, 1995). Molecular analysis showed that triggering of CR2 by CD23 or anti-CR2 antibodies increased the IL-4 induced germline ε transcription levels and produces a synergistic increase in the expression of ε transcript induced by T cells (Henchoz *et al.*, 1994). Further to that, Bonnefoy *et al.* (1993) demonstrated that a subset of anti-CR2 antibodies and soluble CD23 can promote the rescue of germinal center B cells from apoptosis, extending the previous observation of Liu *et al.* (1991) that soluble CD23 in synergy with IL-1 can rescue the B cell centrocytes in germinal centers from programmed cell death and suggesting that the CD23-CR2 interaction is responsible for this effect of soluble CD23.

Bacon *et al.*, 1993, showed that CR2 expressed on basophilic cells is involved in histamine release triggered by CD23 and anti-CR2 antibodies, suggesting that CD23/CR2 pair acts at another stage of the allergic reaction by regulating the effector cell. The interaction of CD23 and CR2 was also reported to play other functions such as mediating the homotypic aggregation of B lymphocytes (Bjorck *et al.*, 1993). The role of CD23-CR2 interaction in B cell aggregation or in binding of B cell to FDC, rather than B cell to T cell, is signified by the fact that both, B and FD cells express CD23 and CR2, whereas

T cells are negative or express low amount of these molecules. This is somewhat contrasted by the recent finding that $CD4^+$ T lymphocytes in patients with allergic asthma were CD23 positive and that the expression of CD23 in these cells was induced with either an allergen or recombinant IL-4 (Gagro and Rabatic, 1994). Sutton and Gould (1993) have proposed a model for the cell-cell interaction mediated by CD23 and CR2 in which the two major signal transduction pathways mediated by the membrane IgM and CD19 are synergistically related to each other (Fig.1-4). Taken together, the presented evidence suggests an important biological role for CD23-CR2 interaction. The clarification of this will require still further studies, some of which are presented in this thesis.

1.4. FcγRII/CD32: The low affinity receptor for IgG

1.4.1. Diversity of Fcγ receptors

The Fcγ receptors (FcγRs) are a distinct subclass within the Ig superfamily defined for their capacity to bind the Fc fragment of IgG. The FcγR gene family consists of 8 members transcribed into at least 12 different mRNAs, and although not all the corresponding proteins have yet been identified, the diversity of the FcγR family is quite remarkable: there are 3 classes of FcγR genes (I,II,III), each class is represented by 2 or 3 different transcripts (a,b,c) encoding for 1-3 isoforms (1,2,3) and finally, some of these isoforms are composed of heterogeneous protein subunits (α,β,γ) or require the association of additional subunits (ζ). The molecular analysis of the encoding genes and the translated products has indicated that these molecules have evolved from a common precursor through gene duplication. The homology between the extracellular parts of different FcγR isoforms is very high but the transmembrane and cytoplasmic parts, in contrast, display very little homology, the consequence of which is the diversity of intracellular signalling pathways mediated by FcγRs.

These receptors play several important roles in the immune defence system and some of the effector functions triggered by FcγRs are phagocytosis, endocytosis, antibody-dependent cellular cytotoxicity (ADCC) and release of inflammatory mediators (Unkeless *et al.*, 1988). They are expressed on variety of white blood cells, usually in cell-specific combinations of different isoforms, which makes it very difficult to distinguish which

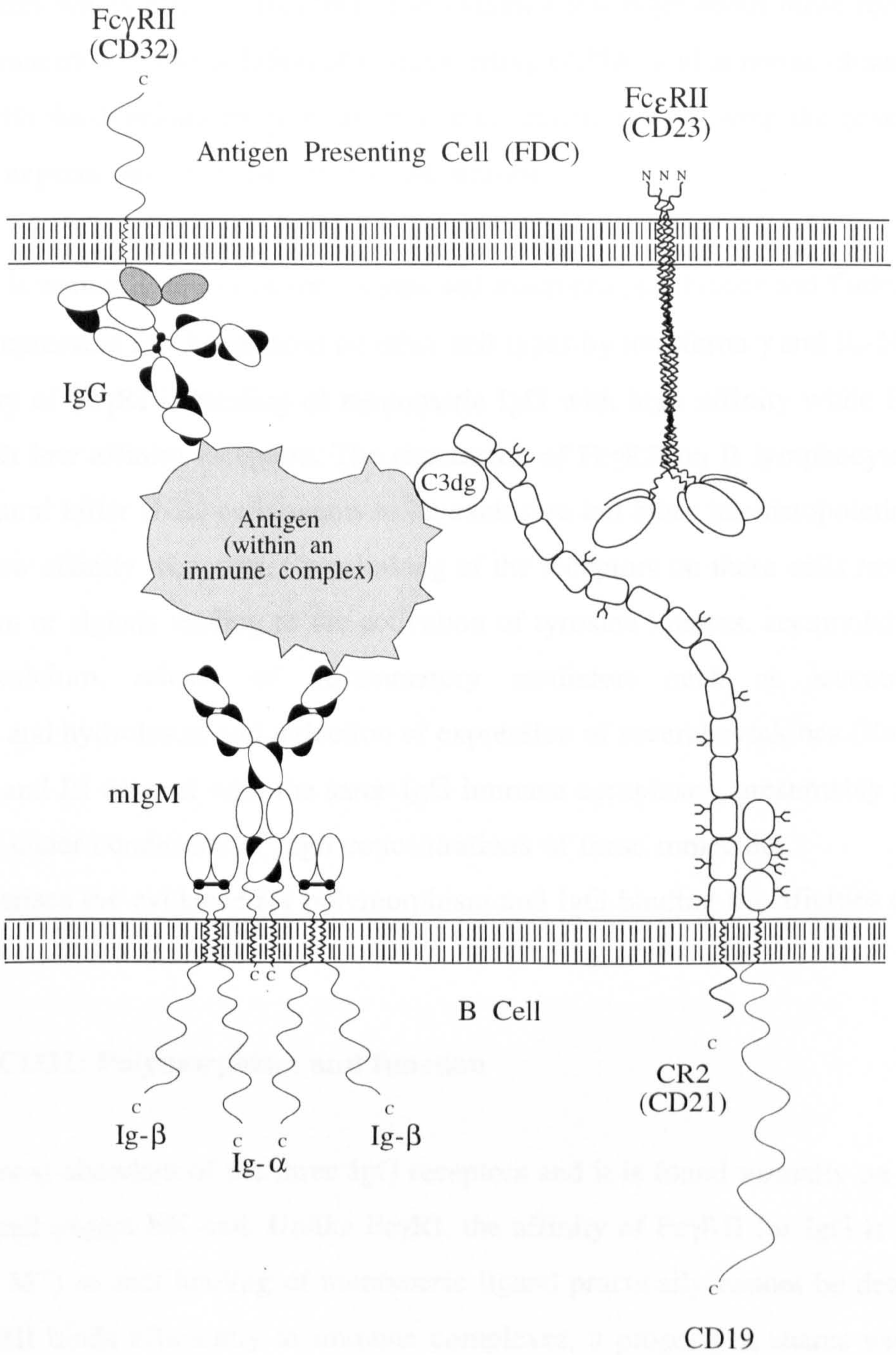


Fig.1-4 CD23-CR2 adhesion pair in the context of FDC-B cell interactions (Sutton and Gould, 1993). Antigen presenting cells such as FDC (follicular dendritic cells) express the Fc receptors that can bind and immobilise antigen within an immune complex and present it to the B cell via the antigen receptor (IgM for, example). CD23 and CR2 may participate in the interaction between the two cells and if the complement is activated may enhance the response of B cell by crosslinking antigen receptor and CR2 (as indicated in the figure). CR2 is closely associated with another membrane protein, CD19 that has great signal transducing capacity and both, CR2 and CD19 are known to synergise with antigen receptor, the consequence of which is lowering the threshold concentration of the antigen required for cell activation and enhancement of the response.

receptor mediates which effector function. The overall knowledge about these receptors has greatly advanced with the isolation of corresponding cDNAs and genomic clones, and in particular with the development of transgenic mice techniques, allowing the generation of strains that express only one particular FcγR isoform.

FcγRI (CD64) is normally found on monocytes and macrophages (Huber and Fudenberg, 1970) but its expression can be induced on other cell types by interferon γ and IL-10. The unique property of FcγRI is binding of monomeric IgG with high affinity while FcγRII and III are both low affinity receptors. The expression of FcγRII on B lymphocytes and FcγRIII on natural killer (NK) cells seems to be exclusive but other haematopoietic cells express both low affinity receptors. Crosslinking of the receptors on these cells results in the transduction of signals leading to the activation of tyrosine kinases, accumulation of intracellular calcium, release of inflammatory mediators such as leucotrienes, prostaglandins and hydrolases, and induction of expression of several cytokines (Ravetch, 1994). FcγRII and III interact with the same IgG immune complexes, presumably acting synergistically under conditions of high concentrations of these molecules.
Table 4 summarises the evidence for polymorphism and IgG binding specificities of Fcγ receptors.

1.4.2. FcγRII/CD32: Polymorphism and function

FcγRII is the most abundant of the three IgG receptors and it is found virtually on every FcγR bearing cell except NK cell. Unlike FcγRI, the affinity of FcγRII for IgG is rather low (Ka $\leq 10^7$ M^{-1}) so that binding of monomeric ligand practically cannot be detected. However, FcγRII binds efficiently to immune complexes, a property it shares with the other low affinity receptor for IgG, FcγRIII. The molecular studies of FcγRII have been carried out with the use of several, well characterised monoclonal antibodies to this protein, in addition to the heterologous expression of cDNA clones in several cell lines (Ravetch and Kinet, 1991).

In human system, FcγRII is encoded by a minimum of 3 genes giving rise to 6 distinct transcripts (Qin *et al.*, 1990). The FcγRIIB gene gives rise to 3 transcripts (b1,b2,b3) as

Table 1-4: Diversity of human Fcγ receptors; nomenclature and IgG binding characteristics.

Nomenclature	Other names	Gene transcripts	Affinity for IgG (Ka)	Specificity for IgG isotypes
huFcγRI	FcγRI, CD64	4 (a,b1,b2,c)	10^8 - 10^9	IgG1=IgG3> IgG4>>IgG2
huFcγRIIA	FcγRIIa, CD32	2 (a1,a2)	< 10^7	IgG1=IgG3 >>IgG2,IgG4
huFcγRIIB	FcγRIIb, CD32	3 (b1,b2,b3)	< 10^7	IgG1=IgG3 >>IgG2,IgG4
huFcγRIIC	FcγRIIa, CD32	1 (c)	< 10^7	IgG1=IgG3 >>IgG2,IgG4
huFcγRIIIAα	III-2, CD16	1 (a)	< 10^7	IgG1=IgG3 >>IgG2,IgG4
huFcγRIIIB	III-1, CD16	1 (b)	< 10^7	IgG1=IgG3 >>IgG2,IgG4

a result of alternative splicing, while FcγRIIA gene encodes for two proteins (a1,a2) and FcγRIIC for one. The further diversity among this receptor isoforms is provided by a single point mutation in FcγRb1 (tyrosine at the position 12 is replaced by aspartic acid), giving rise to a variant which is in the already complex nomenclature of FcγRs given the distinction by an "*" (FcγRIIb1*). The homology between the extracellular parts of different FcγRII isoforms is very high (> 95 %) so that no monoclonal antibody that would distinguish between them has yet been generated. The FcγRIIB isoforms are preferentially expressed in lymphocytes, whereas IIA and IIC isoforms are expressed in neutrophils; monocytes express all three classes. The affinity of the FcγRII isoforms for different IgG isotypes is largely common and IgG3 and IgG1 are bound with much higher affinity than IgG2 and IgG4 (Warmerdam *et al.*, 1993; Parren *et al.*, 1992).

FcγRII is a 40 kDa membrane protein which can be cleaved close to the cell surface to generate soluble forms which circulate in biological fluids. However, Delasalle *et al.*, 1992, presented the evidence for the presence of two different mRNAs for FcγRII, one of which encoded the membrane bound receptor and the other the soluble form of the protein. The ratio between the two forms was 1:1.5 in the favour of the mRNA encoding the membrane expressed receptor. The addition of TNF α (tumour necrosis factor) to the culture of human Langerhans cells modified this ratio to 1:0.6 in favour of the soluble receptor encoding mRNA, thus suggesting a role for TNF α in modifying the expression of FcγRII (or, at least class A) at the mRNA level. The soluble forms of FcγRII, as well as the other two classes of Fcγ receptors (I and III) display the capacity to bind IgG in the similar manner as the membrane bound forms of the receptor (Teillaud *et al.*, 1994).

The ability of FcγRII to trigger phagocytosis, endocytosis, influx of Ca^{2+}, capping and cytokine release after crosslinking has been confirmed in both, human and murine system. The FcγRII signalling pathway is linked to G proteins and may take different patterns resulting in different cellular responses (Unkeless, 1989). Neutrophil degranulation and superoxide production can be triggered by anti-FcγRII antibodies, while monocyte FcγRII activation results in an internal increase of Ca^{2+} ions. The human FcγRII may also serve as a bridge between NK cells and target cells, as shown by the evidence that anti-FcγRII

antibody mAb IV.3 and its Fab fragment partially block NK cytotoxic activity against the target cells (Perl *et al.*, 1986).

FcγRII is generally used as the model receptor for antibody/antigen complexes on antigen presenting cells for various studies. In these studies, FcγRII positive cell line is often used as the antigen presenting cells, to study the effects of ligation of different B cell antigens, alone or in combination with the antigen receptor ligation. Although the name "FcγRII" was used in this section of the Thesis, for the purpose of correct nomenclature of Fcγ receptor isoforms, this receptor also has a CD name, CD32 (initially CDw32). This name will be used in the rest of this thesis, to emphasise the distinction from the alternative name for CD23, FcεRII.

1.5 Objective of the study

This study aimed to further asses the interaction of CD23 with CR2 by applying different recombinant and native forms of both proteins and look at the functional consequences of this interaction in the process of B cell activation. One of the major objectives in this framework was to find out whether soluble CD23 can bind CR2. Soluble CD23 has been shown capable of promoting the survival and differentiation of germinal center B cells and it has been suggested that this is achieved through its interaction with CR2 on these cells. However, no published evidence has yet been presented that would confirm this hypothesis and, in fact, two reports indicated that recombinant soluble CD23 failed to bind CR2. The role of soluble CD23 as an autocrine growth factor for activated B lymphocytes has been also questioned following a report that recombinant form of the protein did not display the activity that has been ascribed to the native soluble fragments.

The laboratory in which the current work was conducted has developed an experimental approach to the study of CD23 that emphasises the significance of the relationship structure-function of this protein. The structural requirements and the stoichiometry of the interaction of CD23 with IgE could be quoted in justification of such an approach. Thus, it was intended in this study to generate a recombinant form of CD23 that could structurally resemble the native soluble protein and to test its capacity to bind CR2. Also, it was intended to generate a recombinant form of CR2 and asses its ability to bind CD23;

this was of the particularly great interest in this work with respect to the published reports that had suggested that only certain glycosylation subtypes of CR2 could bind CD23.

The functional implications for CD23-CR2 interaction, notably, the regulation of IgE synthesis, cell-cell adhesion and the rescue of germinal center B cells from apoptosis, have been suggested. This study aimed to further explore the functional aspect of this interaction, in particular in the context of FDC mediated B cell activation. The experimental approach to study this was to create a fibroblast cell line that could mimic FDC with respect to expression of CD23 and an IgG receptor (CD32) and to look at the effect of CD23 on antigen receptor induced stimulation of B cells. Ultimately, the interaction of CD23 with CR2 has been focused on as the molecular basis for stimulatory activity of both proteins; in addition, a possible synergistic interaction of CD23 with the low affinity receptor for IgG (CD32) on antigen presenting cells has been studied.

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

2.1.1. Reagents and enzymes

All restriction and DNA modification enzymes were from New England Biolabs (Hitchin, UK); DNA sequencing kit was from Promega (Southampton, UK); the reagents IPTG (isopropyl-β-D-thiogalactopyranoside), guanidine chloride, glutathione, OGP (octyl-β-D-glucopyranoside), and TLCK (L-1-chloro-3-[tosylamido]-7-amino-2-heptanone) were all from Sigma (Poole, UK); EDC (1-ethyl-3-(dimethylaminopropyl)-carbodiimide-HCl) was from Pierce (Chester, UK); POPC (1-palmitoyl-2-deoyl-*sn*-glycero-3-phosphocholine) was from Avanti Polar Lipids (Alabaster, USA); DiOC18 (3,3'-dioctadecyloxacarbocyanine perchlorate) was from Molecular Probes (Eugene, USA); protein A and Protein G affinity gels, and Affigel-10 were from BioRad (Hemel Hempstead, UK); ECL reagents for Western blotting, [^{125}I]-NaI and [^{3}H]-thymidine were from Amersham (Little Chalfont, UK); GlycoTrack reagent was from Oxford Glycosystems (Abingdon, UK) and the DOTAP cell transfection reagent was from Boehringer (Mannheim, Germany). The tissue culture media and reagents were purchased as follows: Dulbecco's Modified Eagle's Medium, Iscove's Modified Eagle medium, Minimal Essential Medium α-modification, RPMI 1640, Foetal Bovine Serum, Phosphate Buffered Saline (PBS), Trypsin-EDTA solution, 100 mM glutamine solution, Penicillin-Streptomycin solution, HAT and HT supplements, Percoll and mitomycin C were from Sigma; Hybridoma SFM medium, SF 900-II, geneticin-sulphate (G418), interleukin 4 (IL-4), and versene solution were from Gibco (Paisley, UK); sheep red blood cells (SRBC) were purchased from TCS (Buckingham, UK), lymphoprep was obtained from NYCOMED Pharma (Oslo, Norway). Most of the other chemicals and general reagents not mentioned here were purchased from Sigma.

2.1.2. Plasmids

The plasmid pET-5a was purchased from Novagen (Madison, USA); pUC18-CD23 was obtained from Dr. J. Yodoi (Kyoto University, Kyoto, Japan); plasmid BlueScript was from Stratagene (Cambridge, UK); pEE12 was obtained from Dr. R.J. Owens (Celltech, Slough, UK) and pRTK3 was obtained from Dr. A. Reith (Ludwig Institute, Middlesex, UK).

2.1.3. Antibodies

Anti CD23 antibodies: MHM6 was initially obtained from Dr. J. Gordon (University of Birmingham, Birmingham, UK) and was later purified from hybridoma culture; mAb25 was obtained from Dr. J.Y. Bonnefoy (Glaxo Institute, Geneva, Switzerland) and BU38 was purchased from Binding Site (Birmingham, UK).

Anti CR2 antibodies: OKB-7 was from Orthodiagnostics (High Wycombe, UK); HB5 was purified from hybridoma culture; BU32 and BU36 were from Binding Site (Birmingham, UK).

Other antibodies: mAb IV.3 (anti-CD32) was from Medarex, (Annandale, USA), DAA.4 (anti-IgM) was obtained from Dr. D.T. Fearon (Cambridge), HD37 (anti-CD19), negative controls IgG1 and IgG2a/b and all secondary antibodies were from Dako (Glostrup, Denmark).

2.1.4. Cells and cell lines

B cells: RPMI 8866, RPMI 8226 and Raji cells were purchased from ATCC (American Type Cell Culture, Rockville, USA); tonsils for isolation of B cells were obtained from Royal National Hospital, London, UK.

Other cells: Insect cells Sf9 were obtained from ECACC (European Collection of Animal Cell Cultures, Salisbury, UK); LTK^- fibroblast cell line transformed with human FcγRII cDNA was obtained from Dr. D.T. Fearon (Cambridge).

Hybridomas: MHM6 producing hybridoma cell line was obtained from Dr. J. Gordon (Birmingham) and HB5 producing hybridoma cell line was purchased from ATCC.

2.1.5. Common buffers and solutions used in this work

PBS: Phosphate-buffered saline, 11.9 mM with respect to phosphate and 140 mM with respect to chloride; 1 l of solution is prepared by dissolving in distilled water 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24g KH_2PO_4 and adjusting pH to 7.4.

TBS: Tris-buffered saline, 25 mM with respect to Tris and 140 mM with respect to phosphate; 1 l of solution is prepared by dissolving in distilled water 8.0 g NaCl, 0.2 g KCl and 3.0 g Tris-base, and adjusting the pH to 7.5.

HBS: HEPES-buffered saline, 20 mM with respect to HEPES and 140 mM with respect to NaCl; 1 l of solution is prepared by dissolving in distilled water 4.77 g HEPES free acid and 8.19 g NaCl, and adjusting the pH to 7.2.

TE: Tris-EDTA; 10 mM Tris pH 8.0, 1 mM EDTA.

TAE: Tris-acetate-EDTA DNA electrophoresis buffer; 40 mM Tris, 1 mM EDTA, pH adjusted to 8.0 with glacial acetic acid. The solution is prepared as a 50X stock.

SDS-PAGE buffer: 25 mM Tris, 250 mM glycine, 0.1 % SDS (pH should be about 8.3); 1 l of 5X stock buffer solution is prepared by dissolving in distilled water 30.25 g Tris base, 187.5 g glycine and 10 g SDS.

Native PAGE buffer: 25 mM Tris, 25 mM boric acid (pH should be about 8.7); the solution is prepared as a 5X concentrated stock.

Western blotting buffers: anode buffer contains 0.3 M Tris pH 10.4 and 20 % methanol; cathode buffer contains 25 mM Tris pH 9.4, 40 mM ε-aminocaproic acid and 20 % methanol.

Blocking buffer for Western blotting: 5 % nonfat dried milk in PBS, with 0.02 % sodium azide.

Cell lysis buffer: 1.4 % OGP in PBS, with protease inhibitors added. For isolation of CD23 the inhibitors were: 2 mM PMSF, 0.2 mM TLCK, 20 mM iodoacetamide, 10 mM benzamidine hydrochloride, 50 mM ε-aminocaproic acid.

Buffer for coupling antibodies to Affigel-10: 0.1 M MOPS, 0.3 M NaCl.

Affinity column elution buffer: for elution of antibodies from protein A or protein G columns 0.1 M glycine pH 2.5 was used; for elution of native CD23 from anti-CD23 affinity columns 0.2 M glycine with 0.2 % OGP and 0.2 mM TLCK was used.

Binding buffer for FACs analysis: for antibody binding to cell antigens PBS containing 2 % BSA and 0.1 % sodium azide was used; for CD23/CR2 binding the buffer used was HBS with 1 % BSA, 2 mM $CaCl_2$ and 0.1 % sodium azide.

Coomassie staining and destaining solutions: 0.25 % Coomassie Brilliant Blue stain in 60 % methanol, 10 % acetic acid; destaining solution was 60 % methanol, 10 % acetic acid.

Silver staining and reducing solutions: 100 ml of silver staining solution is prepared by dissolving 0.8 g $AgNO_3$ in 2 mM NaOH solution to which 1.4 ml of 35 % ammonia solution is added; the reducing solution is prepared from 0.5 ml of 1 % citric acid and 52 µl of formaldehyde added to 100 ml of distilled water.

RE buffers: all restriction enzyme buffers used in this work were those supplied with the enzymes by the manufacturer.

Ligation buffer: 50 mM Tris, 5 mM $MgCl_2$, 5 mM DTT and 0.5 mM ATP; the blunt ends ligation buffer is described in the text.

Sodium azide solution: 20 % sodium azide solution was prepared and used as a 1000X stock (where the purpose was preventing bacterial contamination of solutions) or 200X stock (where the purpose was preventing the endocytosis of cell surface antigens).

2.2. Recombinant DNA techniques

2.2.1 Transformation of *E. coli* and large scale bacterial culture

The competent *E. coli* cells (strains HB101 or TG1 for general purpose plasmid isolation or otherwise as stated in the text) were transformed in the total volume of 200 µl by addition of 1-10 µl of plasmid DNA in TE buffer (the amount of DNA used was typically 0.1 µg) and incubation on ice for 30 min. After that the cells were exposed to the temperature shock (42 °C/2 min) and returned to ice for 5 min, followed by addition of 0.8 ml of LB medium (1 % bactotryptone, 0.5 % bacto-yeast extract, 1 % sodium chloride, pH adjusted to 7.0 with 1 M NaOH) and incubation at 30 °C for 45 min. An aliquot of 100 µl was spread over agar plates (L-agar/ LB medium) containing 50-100 µg ampicillin and the plates were incubated for up to 24 h, by which time the bacterial colonies become visible. Several colonies were expanded onto new agar plates and after overnight incubation these cultures were used for inoculation of fresh LB medium (containing ampicillin). This was usually done in 2 l sterile laboratory flasks accommodating 500 ml of culture, which was incubated for 12-18 h at 30 °C with constant shaking.

2.2.2. Large scale isolation and purification of plasmid DNA

For the large scale isolation and purification of plasmid DNA the method of lysis of bacterial cells by alkali was used followed by two rounds of centrifugation in CsCl gradient. The bacterial pellet from 500 ml culture was resuspended in 10 ml of solution 1 (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA) containing 5 mg/ml lysozyme and incubated at room temperature for 5 min. 20 ml of solution 2 (0.2 M NaOH, 2 % SDS) was added and the tube gently inverted several times to mix the contents, followed by 10 min incubation on ice. 15 ml of solution 3 was added (5 M potassium acetate pH 4.8), the tube inverted sharply several times until the white precipitate from denatured proteins forms and incubation on ice continued for another 10 min. The mixture was filtered through 2 layers of sterile muslin and centrifuged for 30 min at 10000 rpm in a Sorvall GSA rotor at 4 °C. The supernatant was warmed up to room temperature and 0.6

volumes of isopropanol was added, followed by 15 min incubation at room temperature. The DNA was recovered by centrifugation in a Sorvall S-34 rotor at 12000 rpm and washed with 70 % ethanol. Finally, the DNA pellet was dried on air and dissolved in TE buffer.

The plasmid DNA was purified by centrifugation in CsCl density gradient which was prepared by dissolving 1 g of CsCl and 80 µl ethidium bromide solution (10 mg/ml) per each ml of DNA solution and brief centrifugation at 8000 rpm to remove the insoluble material. The solution was transferred into Beckman Quick-Seal tubes and centrifugation performed in Ti65 rotor at 60000 rpm for 24 h. The plasmid DNA band was located in the gradient under UV light and extracted from the tube with a needle and syringe. The centrifugation in CsCl gradient was repeated once more if the DNA was to be used for transfection of cells. After extraction of ethydium bromide by chloroform the sample was extensively dialysed to remove CsCl and DNA precipitated by ethanol.

2.2.3. General DNA techniques

The restriction enzymes and DNA modification enzyme. reactions were performed according to the manufacturer's instructions. Typically, the restriction digestion was carried out with 5-10 units of enzyme activity per 1 µg of plasmid DNA for 2-3 hours at 37 °C; in the case of partial digestion lower concentration of the enzyme was used and the optimal incubation time determined by pilot experiments. The ligation of blunt DNA ends was performed at 16°C with 2-5 Weiss units of bacteriophage T4 DNA ligase (high concentration) in the buffer: 50 mM Tris, 5 mM $MgCl_2$, 5 mM DTT, 10 mM hexaminocobalt chloride, 0.5 mM spermidine and 100 µM ATP, over a period of 12 h. Prior to ligation, the 5'- ends of the linearised plasmid DNA were dephosphorylated by alkaline phosphatase treatment (1 h/37 °C) following the completion of the digestion reaction. PCR was performed using the appropriate oligonucleotide primers and plasmid DNA as the template (for generation of given PCR fragment) or cell lysate as the source of template (for screening the transformants). In most cases the PCR conditions were: denaturation, 1 min / 94 °C; annealing, 2 min / 55 °C; polymerisation, 2 min / 74 °C; 25 cycles were usually sufficient to generate the required amount of the desired DNA

fragment. Where necessary, the Mg^{2+} concentration was varied to achieve the maximum yield. The DNA was routinely concentrated by ethanol or iso-propanol precipitation. DNA sequencing was performed according to the protocol supplied with the Promega sequencing reagents.

2.2.4. DNA electrophoresis

DNA electrophoresis was performed on 0.8-1.2 % agarose gels with TAE electrophoresis buffer (Sambrook *et al.*, 1989) containing 0.5 µg/ml ethidium bromide. Depending on purpose, small (analytical) or large (preparative) gels were used. The DNA size markers used were phage λ DNA fragments obtained by digestion with *Sty I* and the corresponding sizes of fragments are: 19.3, 7.74, 6.22, 4.25, 3.47, 2.69, 1.88, 1.49, 0.92, 0.42 and 0.014 kb. Following electrophoresis, the DNA bands were located in the gel by illumination with UV light and the DNA fragment of interest was recovered and purified from the gel using the Geneclean kit (La Jolla, USA), according to the manufacturer's instructions.

2.2.5. Construction of the expression vector pRR28

A cDNA fragment coding for the entire extracellular sequence of CD23 was amplified by PCR using pUC-18 (CD23 cDNA storage plasmid) as the template and the primers 5'-CAGGATCCCATATGGACACCACACAGAGT-3' (forward) and 5'-GCGGATCCTATTATCAAGAGTGGAGAGG-3' (reverse) with the both primers designed to generate a new cleavage site for the restriction enzyme *BamHI* and the forward primer carrying a new site for *NdeI*. The PCR fragment was blunt-ended using T4-DNA polymerase, purified by electrophoresis and ligated into *SalI, SacII* cleaved and blunt-ended plasmid Blue Script. The large *BstEII-SacII* fragment of the PCR insert was then replaced within the Blue Script plasmid with the same insert of genomic DNA from the original CD23 plasmid and the remaining parts of the insert on both sides sequenced. The plasmid was then digested with *NdeI* and *BamHI* and the CD23 DNA carrying fragment subcloned into the *E. coli* expression vector pET-5a linearised with the same enzymes. Thus, the *NdeI* site containing ATG codon was restored and the following codon encodes for the first amino acid of the extracellular sequence of CD23.

2.2.6. Construction of the expression vector pML95

The plasmid pML95 was constructed from the mammalian cell expression vector pEE12 into which the entire CD23 coding sequence was inserted as described below. pUC-18 containing the cDNA for CD23 was cleaved with restriction enzymes *SalI* and *RsaI* and the 1.25 Kb *SalI-RsaI* fragment isolated and purified. This fragment, which carries the CD23 cDNA, was then subcloned into the plasmid pBSM13$^-$ previously cleaved with the same enzymes, followed by isolation of a *HindIII-EcoRI* fragment (partial digestion with *HindIII* was necessary to preserve the *HindIII* site within the CD23 cDNA). The *HindIII-EcoRI* fragment was finally subcloned into the expression vector pEE12 which had been previously cleaved with *HindIII and EcoRI*. The transcription of the sub-cloned insert is driven by hCMV (human megalovirus) promoter and the plasmid contains the glutamine synthetase gene for selection of stable transformants and the ampicillin resistance gene for amplification in bacteria.

2.3. Analytical methods

2.3.1. Polyacrylamide gel electrophoresis (PAGE) of proteins

a) SDS-PAGE: electrophoresis of proteins under denaturing conditions

The electrophoresis of proteins in the presence of SDS was performed essentially by the method of Laemmli, 1970. The polyacrylamide gels (110 mm x 80 mm x 1 mm) were prepared from 10 ml of the mixture according to the Table 2-1, which was poured in to the pre-set cassette and left to polymerize for 20-30 min. On the top of the resolving gel of given concentration 1.5 ml of the stacking gel mixture (5 % acrylamide) was poured and 12 teeth comb inserted, generating slots of the maximum loading capacity of 25 µl. The protein samples (0.1-30 µg, depending on the detection method applied) were mixed with the sample-loading buffer (50 mM Tris pH 6.8, 2 % SDS, 10 % glycerol, 0.1 % bromphenol blue) with or without 100 mM DTT (dithiothreitol) and boiled for 3 min to denature the proteins. Electrophoresis was performed in the electrophoresis apparatus model AE-6450 (ATTO Corporation, Japan) in the presence of running buffer (section

2.1.5), at 120 V for approximately 2 h, after which the gel was used for detection of proteins as described below or for transfer of proteins to nitrocellulose filter and immuno-detection of the specific protein. Parallel to the samples the standard molecular weight protein markers were applied (pre-stained protein markers from Amersham, 14.3-200 kDa and 2.35-46 kDa).

Table 2-1: Solutions for preparing 10 ml of resolving SDS-polyacrylamide gels of the given concentration (the volumes are in ml)

COMPONENT	6 %	8 %	10 %	12 %	15 %
Distilled water	2.6	4.6	4.0	3.3	2.3
30 % acrylamide mix*	1.0	2.7	3.3	4.0	5.0
1.5 M Tris (pH 8.8)	2.5	2.5	2.5	2.5	2.5
10 % SDS	0.1	0.1	0.1	0.1	0.1
10 % amm. persulphate	0.1	0.1	0.1	0.1	0.1
TEMED	0.008	0.006	0.004	0.004	0.004

* 30 % acrylamide solution contains 29 % acrylamide and 1 % N,N'-methylenebisacrylamide

Table 2-2: Solutions for preparing 2 ml of 5 % stacking gel for SDS-PAGE

COMPONENT	VOLUME IN ML
Distilled water	1.4
30 % acrylamide mix	0.33
1.0 M Tris (pH 6.8)	0.25
10 % SDS	0.02
10 % amm. persulphate.	0.02
TEMED	0.002

b) Native PAGE: electrophoresis of proteins under native conditions

This was performed as for the SDS-PAGE with the following exceptions. The gel preparing, sample loading and running buffers did not contain any SDS and the Tris-borate buffer was used instead of Tris-glycine. The protein sample was applied onto the gel directly, without boiling and the separation of proteins was usually performed on polyacrylamide gradient (5-20 %) instead of homogenous gels. The gradient gels were prepared using a gradient maker consisting of two chambers connected with a tap, and a peristaltic pump to transfer the mixture into the pre-set cassette into which a needle was inserted. Table 2-3 contains the details for preparation of polyacrylamide gradient gels.

Table 2-3: Solutions for preparing 10 ml of native PAGE gel with linear gradient of polyacrylamide 5-20 % (ml):

COMPONENT	5 % gel	20 % gel
Distilled water	2.87	0.4
30 % acrylamide mix	0.83	3.3
1.0 M Tris pH 8.7	1.25	1.25
10 % ammonium persulphate	0.050	0.050
TEMED	0.002	0.002

2.3.2. Detection of proteins in gels

Following the electrophoresis, the proteins were detected in gels either by staining in Coomassie Brilliant Blue solution for 2 or more hours and removing the excess of the dye by incubation in the destaining solution, or with staining by silver nitrate method. The silver staining method was performed by first incubating the gel in 20 % TCA (trichloroacetic acid) for 2 or more hours followed by 2 washes in 50 % methanol (30 min each) and 2 washes in distilled water (20 min). The gel was then submerged into the silver staining solution (paragraph 2.1.5) for 10-15 min on a shaking platform, followed by 2 washes in distilled water (5 min) and finally transferred to the reducing solution in which it was incubated until the appearance of protein bands. In the case of [^{125}I]-labelled proteins the gel was dried on 3MM Whatman filter paper and exposed to X-ray film (Fuji) at -70 °C for several hours to overnight.

2.3.3. Western blotting

Following electrophoresis the proteins were transferred to nitrocellulose filter in a semi-dry blotter apparatus (20 V / 20 min) and the remaining free sites on the filter blocked

by incubation in 5 % nonfat dry milk solution in PBS, for 2 h at room temperature or overnight at 4 °C (sodium azide added), with constant agitation on a shaking platform. The filter was briefly washed in PBS and antibody solution added (most of the commercial antibodies were used at the dilution 1:1000, or otherwise, at the concentration 0.05-0.5 µg/ml, in PBS) and the filter incubated in the antibody solution for 2 h at room temperature or overnight at 4 °C, followed by 3 washes in PBS containing 0.02 % Tween 20 (10 min each). After that the filter was incubated with either secondary antibody - peroxidase conjugate or with streptavidin - peroxidase complex in the case where the primary antibody was biotinylated. In either case the nitrocellulose was incubated in the solution for 2 h at room temperature, washed 3 times and the immunoreactive proteins detected by developing the peroxidase reaction. For this, the ECL Western blotting reagents from Amersham were used or the conventional method was applied, utilizing the chromogen diaminobenzidine tetrahydrochloride.

2.3.4. ELISA Immunoassay

The ELISA immunoassay was performed in 96-well Nunc-immuno plates by coating the plates with the first protein at the concentration 2 µg/ml in 100 mM sodium carbonate buffer pH 9.6, for 2 h at room temperature or overnight at 4 °C, followed by blocking the unoccupied sites with 2 % nonfat dry milk solution in the same buffer. The wells were then briefly washed with PBS, 0.02 % Tween 20, and the second protein was added at serial dilution (2-fold or 10-fold). Again, the plates were incubated for 2 h, washed 3 times and in the case where the second protein was primary antibody, the secondary antibody - peroxidase conjugate was added; where the second layer was another protein the primary antibody was added followed by incubation with secondary antibody - peroxidase conjugate. After the incubation and washing as above the peroxidase reaction was developed using the chromogen substrate tetra-methylbenzidine and the absorbance read at 630 nm, in a multi-channel absorbance reader (Titertek Multiscan Plus, from ICN Flow). In some cases, where higher sensitivity was required, an equal volume of 3 M sulphuric acid was added to the final reaction and the absorbance read at 450 nm.

2.3.5. Isolation and purification of recombinant CD23 from *E. coli*

The bacterial cells from 1 l of minimal medium M9 were harvested 6 h following the induction with 0.4 mM IPTG and the inclusion bodies with accumulated recombinant CD23 extracted from the cell pellet by the modified procedure of Bohmann and Tjian, 1989. The cell pellet was resuspended in 72 ml of solution 1 (10 mM Tris pH 7.9, 25 % sucrose, 100 mM KCl, 2 mM DTT, 2 mM PMSF) and 1/4 vol. (18 ml) of the solution 2 (300 mM Tris pH 7.9, 100 mM EDTA, 4 mg/ml lysozyme) was added. Following 10 min incubation on ice, the equal volume (90 ml) of solution 3 (1 M LiCl, 20 mM EDTA, 0.5 % Nonidet NP-40) was added and the suspension mixed and sonicated (Soniprep 150). The inclusion bodies were pelleted by centrifugation at 10000 rpm for 10 min, resuspended in 200 ml of solution 4 (10 mM Tris pH 7.9, 0.1 mM EDTA, 0.5 mM LiCl, 0.5 % Nonidet NP-40, 1 mM DTT, 1 mM PMSF) and the sonication repeated. After centrifugation (as before) the pellet was resuspended in 200 ml of solution 5 (10 mM Tris pH 7.9, 0.1 mM EDTA, 2 % Nonidet NP-40, 1 mM DTT, 1 mM PMSF), sonicated again, centrifuged and resuspended in 200 ml of solution 6 (10 mM Tris pH7.9, 0.1 mM EDTA, 0.5 % Nonidet NP-40, 1 mM DTT, 1 mM PMSF). Finally, the sonication was repeated once more and the remaining pellet frozen at -70 °C. For isolation of recombinant CD23 from the inclusion bodies, the pellet was dissolved in 5 ml of 6 M guanidine-HCl (GnCl), 20 % sucrose, 10 mM DTT and incubated at room temperature for 1 h. CD23 was then purified by gel-filtration on a Sephacryl S-100 column (Pharmacia) with 6 M GnCl as the running buffer. The fractions were analyzed for CD23 by electrophoresis and Western blotting, and the positive fractions pooled and concentrated by ultrafiltration (Amicon).

2.3.6. Renaturation of recombinant CD23

For the renaturation of insoluble, inactive CD23 isolated from the bacterial culture by GnCl extraction and purified by gel-filtration, several protocols were applied, based on different oxidation conditions. The optimal protocol for the refolding of CD23 was shown to be similar to that for the protease papain, as described by Taylor *et al.*, 1992. The protein stock solution (1 mg/ml, in 6 M GnCl) was concentrated further 10-fold by ultrafiltration and DTT was added to the final concentration of 10 mM. Following 1 h

incubation at 37 °C the sample was diluted 10-fold in 0.5 M Tris-acetate pH 8.6 containing 6 M GnCl and 0.1 M oxidised glutathione, and stored at 4 °C for 24 h. The sample was diluted further 100-fold in 100 mM Tris-acetate buffer pH 8.0, 2 mM $CaCl_2$, 3 mM cysteine and incubated for 36 h at 4 °C. Finally, CD23 was concentrated 1000-fold by ultrafiltration, with a gradual change of the refolding buffer for TBS pH 7.5, 2 mM $CaCl_2$, 0.2 mM TLCK (L-1-chloro-3-[4-tosylamido]-7-amino-2-heptanone).

2.3.7. [^{125}I]-labelling of recombinant CD23

100 µg of recombinant CD23 was labelled with [^{125}I]-NaI by mixing 100 µl of protein sample (1 mg/ml), 100 µl of 0.5 M sodium phosphate buffer pH 7.5, 1 mCi of [^{125}I]-NaI (Amersham) and 200 µl of chloramine T solution (2 mg/ml, in 250 mM sodium phosphate buffer pH 7.5). Following 2 min incubation at room temperature, 400 µl of stop solution (2 mg/ml sodium-metabisulphite, in PBS) and 200 µl of PBS were added. The free label was removed by gel-filtration on a small Sephadex-50 column and the protein containing fractions (1 ml each) combined and frozen. An aliquot of this was used to measure the total radioactivity in a gamma radioactive counter, and the specific radioactivity was measured on the basis of protein measurement by spectrophotometry.

2.3.8. Chemical crosslinking of recombinant CD23

The chemical crosslinking of recombinant CD23 was performed as described by Beavil *et al.*, 1995. The conditions for optimal cross-linking with respect to pH of the buffer, concentration of the protein and the crosslinker and the length of incubation, were tested separately. Typically, the [^{125}I]-labelled CD23 was crosslinked at the protein concentration 2 µg/ml in 20 µl of 25 mM sodium acetate buffer pH 5.0 with the crosslinker 1-ethyl-3-(dimethylaminopropyl)-carbodiimide-HCl (EDC). After 15-30 min incubation at the room temperature the sample was subjected to SDS-PAGE (12 % polyacrylamide gel) under reducing conditions and the dried gel exposed to X-ray film. For crosslinking of CD23 at higher concentrations (50 µg/ml, 0.5 mg/ml) the same conditions were used except that unlabelled CD23 was used and the protein visualised in the gels after electrophoresis by silver or Coomassie staining methods.

2.3.9. HPLC analysis of proteins

For additional purity of CD23 or for analytical purposes (molecular weight determination, Stokes radius measurement) some protein preparations were subjected to HPLC on Superdex-75 column (Pharmacia). The running buffer was TBS pH 7.5 containing 2 mM $CaCl_2$ and 0.2 mM TLCK. The column was equilibrated with the standard protein molecular weight markers, as indicated in the text.

2.3.10. Sucrose gradient centrifugation

The sucrose gradients (5-20 %) in TBS, 2 mM $CaCl_2$, 0.2 mM TLCK were prepared in 12 ml polypropylene tubes (Beckman) using a gradient maker comprising two equal chambers connected with a tap, to which 6 ml of 5 % or 20 % sucrose solution were added and the tap opened (the chamber closer to the pump to which the 5 % solution is added contained a small magnetic stirrer, for rapid mixing of the solution). The mixed solution was poured into the tube via a small glass capillary inserted to the bottom of the tube, so that the incoming sucrose solution of higher concentration was pushing the 5 % solution upwards until reaching the top of the tube.

The protein sample was loaded on the top of each gradient and the centrifugation performed with a Beckman SW 41 rotor at 35000 rpm, at 4 °C. The centrifugation time was calculated from the "K" value of the rotor (given in the catalogue supplied by the manufacturer) adjusted for the actual speed as compared to the maximum speed ($K_{adj.} = K \times [rpm_{(max)}/rpm_{(actual)}]$). The time of centrifugation was usually calculated with respect to the protein with highest $S_{w,20}$ value (sedimentation coefficient) nearly reaching the bottom of the tube at the end of centrifugation; in the equation $K = t \times S$, t represents the time and S the sedimentation coefficient of the protein. For most of the experiments the centrifugation time was between 24 and 36 h.

Following the end of centrifugation, the gradients were fractionated in 50-60 samples (0.2-0.25 ml) and the fractions analyzed for proteins by spectrophotometry (OD_{280nm}). The sedimentation coefficient for CD23 was determined from a calibration curve obtained for

the internal standards and subsequently used to calculate the molecular weight of the protein from the classical Svedberg's equation (described later in the text).

2.3.11. Surface plasmon resonance (SPR)

This was performed in a BIAcore biosensor apparatus (Pharmacia) as follows. The protein of interest was immobilised via primary amine groups to a carboxylated dextran-coated CM5 sensor chip at the concentration 50 µg/ml and the unoccupied sites on the chip blocked with 1 M ethanolamine-hydrochloride. The ligand was then injected at various concentrations and association phase was monitored for 6 min at a flow rate of buffer at 5 µl/min, while the dissociation was monitored for 10-15 min. Between each run the remaining ligand was removed from the surface by repeated pulses of 0.1 M glycine pH 2.5. As the negative control a sensor chip with no first protein immobilised but treated in the same way, was used. Since binding was measured in presence of the constant concentration of the first protein in the flow cell, the association rate constant (k_{+1}) was calculated assuming pseudo first-order kinetics. If R is the signal (in resonance units, RU) dR/dt was plotted against R for each concentration (over the time period in which the binding is not limited by mass transfer). The gradients of these lines were then plotted against the concentration of ligand and k_{+1} obtained from the gradient of this plot (Karlsson *et al.*, 1991). The dissociation rate constant (k_{-1}) was obtained from the gradient of a plot of $\ln(R_0/R_t)$ against time, where R_0 and R_t are the signals at the start of, and during the dissociation phase. The affinity constant for binding was then calculated as k_{+1}/k_{-1}.

2.3.12. Purification of native CD23 from RPMI 8866 cells

The membrane form of CD23 was isolated and purified from the detergent lysates of 10^{11} RPMI 8866 cells similarly as described by Cairns and Gordon, 1990, with one modification. The detergent used was octylglucopyranoside (OGP) instead of Nonidet NP-40. The cells were lysed in the buffer PBS pH 7.4, 1.4 % OGP, 10 mM benzamidine hydrochloride, 20 mM iodoacetamide, 50 mM ε-aminocaproic acid, 0.2 mM TLCK and 1 mM PMSF, for 2 h on ice, followed by 30 min centrifugation at 16000 x g. The

supernatant was applied onto MHM6 anti-CD23 antibody affinity column prepared by binding of 100 mg pure antibody to 20 ml of Affigel-10 (Bio-Rad) and the column washed with 20-30 volumes of lysis buffer before the protein was eluted in 0.2 M glycine, pH 2.3, 0.2 mM TLCK, 0.2 % OGP. In some cases the CD23 preparation purified in this way was passed through a second affinity column prepared by binding of 20 mg anti-CD23 antibody mAb25 to 10 ml of Affigel-10. In either case the CD23 positive fractions were pooled, concentrated and kept frozen.

2.3.13. Preparation of fluorescent CD23-liposomes

The native, detergent solubilized CD23 was incorporated into liposomes together with a fluorescent label by a method similar to that described by Pochon *et al.*, 1992. The phospholipid POPC was dissolved in chloroform at 50 mM concentration (380 mg in 10 ml) and aliquoted in 1 ml samples which were flashed with nitrogen (to remove oxygen) and frozen at -80 °C. The 0.5mM solution of the fluorescent label DioC18 was prepared by dissolving 4.4 mg in 10 ml of dymethylformamide and kept at -20 °C in 0.5 ml aliquots.

For preparation of 1.0 ml of CD23-liposomes 10 µmol (0.2 ml) of POPC and 50 nmol (0.1 ml) of DioC18 were mixed and the solvents evaporated under the flow of nitrogen for 2 h. The sample was further dried in Speed-Vac concentrator and dissolved in 50 µl of 800 mM OGP in PBS, by intensive vortexing. 150 µl of PBS was added (OGP is now 200 mM) followed by addition of 800 µl of 50 mM OGP solution in PBS containing 0.2 nmol of CD23 (approx. 9 µg), to make a ratio protein/lipid 1 : 50000. The mixture was briefly vortexed and incubated on ice for 1 h, followed by filtration through a 0.2 µm filter and dialysed in a Slide-A-lyzer cassette (Pierce) for 3 days, against 20 mM HEPES buffer pH 7.0. The formation of liposomes can be observed by the rapid increase in turbidity of the solution. Sodium azide was added and the CD23-liposome solution was kept at 4 °C, in dark.

CD23-liposomes could not be formed if the detergent for CD23 solubilization was Nonidet NP-40. Even traces of this or other non-dialysable detergents will alter the

CMC (critical micelle concentration) value for OGP, preventing its dialysis and thus, the formation of liposomes.

2.3.14. Purification of native and soluble CR2

The membrane form of CR2 was isolated and purified from 10^{10} RPMI 8226 cells which were lysed in the buffer PBS pH 7.4 containing 1 % Nonidet NP-40 and 1 mM PMSF, for 2 h on ice. The lysates were centrifuged at 16000 x g for 30 min and the supernatant passed through anti-CR2 antibody HB5 affinity column. The column was washed with 30 volumes of lysis buffer and the bound protein eluted with 0.1 M glycine, pH 2.5, 0.1 % Nonidet NP-40.

The recombinant CR2 expressed in insect cells was partially purified from the cell culture supernatants as follows. The insect cell culture supernatants from infected cells were collected usually on day 5 following the infection, centrifuged at 8000 rpm/20 min to remove the cells and cell debris and filtered through 0.4 µm filter. The supernatant was concentrated up to 5 times by Amicon ultra-filtration and extensively dialysed against 10 mM potassium phosphate buffer pH 7.8 (the iso-electric point of CR2 is 7.36). In the meantime a small amount of the ion-exchanger DEAE Sephadex A50 was reconstituted in the same buffer and allowed to equilibrate with the buffer for several hours. The capacity of this ion-exchanger is exceptionally high (> 10 mg/ml), but for the convenience of using a packed column approximately 5 ml wet volume of the matrix was used. The supernatant was applied to the column and the column washed with the running buffer. The elution of the bound proteins was performed with gradient of sodium chloride 0-300 mM, in 10 mM phosphate buffer pH 7.8 and the fractions analyzed for protein content. The protein containing fractions were further analyzed for presence of CR2 by Western blotting.

2.3.15. Production and purification of antibodies

The hybridoma cell cultures were grown in hybridoma-SFM low protein content medium (Gibco) until approximately 2 l of culture was collected. The cells were removed by

centrifugation (5000 rpm/10 min) and the supernatant filtered through 0.2 µm filter. The supernatant was concentrated by ultrafiltration (Amicon) 100-fold and the antibody purified by affinity chromatography on either protein A (IgG2 type antibodies) or protein G (IgG1 type antibodies) column. The chromatography was repeated several times until there was no significant amount of antibody left in the supernatant. The eluted antibody was concentrated to 2-4 mg/ml and stored frozen.

2.3.16. Detection of carbohydrates in glycoproteins

This was performed using GlycoTrack carbohydrate detection kit from Oxford Glycosystems, according to the manufacturer's instructions. The protein sample (0.1-1 µg) was labelled in solution (20 µl) followed by electrophoresis and transfer of the proteins to nitrocellulose filter. Also, a modified protocol for preferential labelling of sialic acid was performed.

2.4. Cell culture techniques

2.4.1. General conditions for growth of cells and cell lines

RPMI 8866: This mature B cell line was used as the source of native CD23. The cells were grown in RPMI 1640 medium supplemented with 10 % FBS (foetal bovine serum), 2 mM glutamine and the antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml). Once confluent, the cells were divided at the ratio 1:10 and culture continued.

RPMI 8226: These plasma B cells were used as the CR2 positive cells in flow cytometry (FACs) assays and also for isolation and purification of native CR2. The growth conditions and maintenance of the cells were same as for RPMI 8866 cells except that the cells were divided at the ratio 1:5 at the passage time.

Tonsillar B cells: The cells were used to study differentiation upon stimulation by crosslinking CD23 and CD32. Once isolated and purified from human tonsils, the cells were kept in complete RPMI 1640 medium supplemented with 10 % FBS and gentamycin

in the incubator (5 % CO_2, 37 °C), at the concentration 10^6 cells/ml, for up to 3 days.

LTK⁻ fibroblast cells: This cell line has been previously transformed with human FcγRII and was used in this work for expression of CD23. The cells were grown in minimal essential medium (α-modification) supplemented with 10 % FBS, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and geneticin/G418 (1 mg/ml). The CD23 transformed cells were TK (thymidine kinase) positive, and were grown in HAT supplemented medium (100 µM hypoxhantine, 0.4 µM aminopterin, 16 µM thymidine). The stable transformants were maintained in HT medium, after selection was complete. The cells were divided every 5-6 days at the ratio 1:10.

Hybridomas: Hybridoma cell lines were used for antibody production. The cells were grown in Hybridoma-SFM low protein content medium from Gibco supplemented with penicillin and streptomycin. The cells were allowed to expand rapidly by dilution with fresh medium (1:3) until sufficient amount of culture supernatant was collected.

Insect cells Sf9: These cells were used for production of soluble CR2 by the baculovirus expression system. The cells were grown in SF 900-II medium supplemented with penicillin and streptomycin, on a shaker. After the infection with recombinant virus the cells were kept in the culture until the cell number fell to 50 % as compared to the initial culture.

2.4.2. Maintenance of cell cultures

All cells were incubated at 37 °C, in the atmosphere of 5 % CO_2, except the insect cells which were grown at the room temperature in the absence of CO_2. The cells were generally grown in tissue culture flasks (T-type) and in some cases in roller bottles on a rolling wheel, while the large scale isolation of membrane CD23 required fermenter production of RPMI 8866 cells (this was done at Pfizer, Kent, UK). The adherent cells were detached from plastics either by trypsin (for maintenance) or by versene (for cell binding assays), or were scraped for preparation of cell lysates. The cells were regularly counted and tested for viability by Trypan Blue staining.

2.4.3. Transfection of cells and selection of stable transformants

The cells were transfected with plasmid DNA double purified by CsCl gradient centrifugation, by the method of lipofection using the transfection reagent DOTAP from Boehringer. The cells were grown in the appropriate cell culture medium until nearly confluent and 5 x 10^5 cells transferred to a new 25 cm^2 tissue culture flask and left to reach 80 % confluence (usually 24 h later). 30 µl of the transfection reagent DOTAP was diluted to 100 µl with HBS, pH 7.2, and 5 µg of plasmid DNA (4 µg of plasmid carrying the gene of interest and 1 µg of selection marker gene carrying plasmid, in the case of co-transfection) was separately also diluted to 100 µl with HBS. The two solutions were mixed and incubated for 10 min at room temperature, after which the mixture was added to 5 ml of culture medium and the cells were incubated with this medium for 12 h. The medium was removed, cells washed with fresh medium and incubated for 24 h in the fresh medium. The cells were left in the incubator for 3 days before they were used in the case of transient expression, or divided in ratio 1:10 and placed in the selective medium in the case of stable expression. The selection took about 2-3 weeks to complete, after which period the single clones were isolated by the method of limited dilution and gradually expanded.

2.4.4. Infection of insect cells with recombinant baculovirus DNA

5 x 10^5 insect cells (Sf9)/ml were infected with the concentrated pure viral stock (3.7 x 10^8 viral particles per 1 ml of the stock) and monitored for viability over a period 1-8 days. After 50 % of the cells were dead (usually 5 days post-infection) the culture was terminated and used as a new viral stock in following infections (approximately 10^6 viral units per 1 ml of the stock). This new stock was routinely used to infect the insect cells in such a way that to the given volume of cell suspension (5 x 10^5 - 1 x 10^6 cells/ml), 1/10 vol/vol of the viral stock was added and the cells incubated until the cell number falls to 50 %.

2.4.5. Cell binding assays and flow cytometry

For binding of CD23-liposomes to CR2 positive cells , 10^6 cells were washed with PBS and resuspended in 200 µl of liposome preparation diluted 5-fold in HBS containing 2 % BSA, 2 mM $CaCl_2$ and 0.1 % sodium azide, and incubated for 2 h on ice. The cells were washed two times in the binding buffer and analyzed for fluorescence in a FACscan apparatus (Becton-Dickinson) against the negative control (liposomes with no CD23). For analyzing the expression of cell surface antigens, 10^6 cells were resuspended in PBS containing 2 % BSA, 0.1 % sodium azide, and the appropriate antibody was added (1:20). Following 1 h incubation on ice the cells were washed twice and incubated with the secondary antibody-FITC conjugate for 1 h on ice. In some cases this was not necessary as the primary antibody was FITC labelled. After washing twice the cells were analyzed by FACs.

2.4.6. AET treatment of sheep red blood cells (SRBC)

The AET (2-aminoeyhylisothiouronium bromide) was prepared as 140 mM solution (1.97 g in 50 ml of distilled water) and pH adjusted to 8.8 with sodium hydroxide (5 M). The solution was filter sterilised before use. The SRBC was aliquoted into two 12.5 ml samples in 50 ml Falcon tubes and to each tube PBS was added to 50 ml. The cells were collected by centrifugation at 12000 rpm/5 min and resuspended in 25 ml of AET solution. The incubation was done at 37 °C for 20 min, after which the cells were collected as previously and repeatedly washed 3-4 times with cold PBS. Finally the cells in each tube were resuspended in 50 ml of complete RPMI 1640 medium with 10 % FBS. This protocol is designed to yield a sufficient amount of AET treated SRBC cells for isolation of B cells from 1 pair of tonsils.

2.4.7. Isolation of human tonsillar B cells

This procedure is an adaptation of the protocol obtained from Dr. J. Murphy (King's College London). The tonsils were first washed in sterile PBS and then cut and minced using scissors and a sterile Petri dish. To release cells two different protocols were

successfully applied. In the first protocol the minced tissue was pressed and squeezed in the Petri dish using a 20 ml syringe plunger and a tea strainer and the released cells collected in 10 ml of RPMI medium. This was repeated 4-5 times until 50 ml of the medium containing cells was collected. In the second protocol the minced tonsillar tissue was placed in a 25 ml glass tissue homogeniser to which 10 ml of RPMI medium was added and the cells released by gently squeezing the tissue. This was repeated several times until the tissue no longer contained mononuclear cells. In either case, the medium with cells was left in the conical 50 ml tube for 5 min and then transferred to a new tube carefully, so that remaining small pieces of the tissue were left behind. 25 ml of Lymphoprep solution (Nycomed) was added per 50 ml Falcon tube and 12.5 ml of the tonsil extract was carefully layered on top of it. The samples were spun at 16000 rpm for 25 min, at the room temperature. The interface between the two media contains the mononuclear cells and was collected in 5-7 ml medium which was then placed in new tube. To wash the cells 50 ml of RPMI medium was added and the cells collected by centrifugation (12000 rpm /5 min), and this was repeated once more. The cell pellet was resuspended in 25 ml of complete RPMI medium and the same volume of freshly prepared AET treated SRBC was added. The contents of the tubes were mixed by gently inverting the tubes and incubated at 4 °C for 30 min. After that the cells were collected by centrifugation at 12000 rpm/5 min and resuspended in 25 ml of complete RPMI medium. This was carefully layered over 25 ml of Lymphoprep solution and centrifugation repeated as above (16000 rpm/25 min, at room temperature. The interface containing B cells was collected in 5-7 ml and the cells washed once with RPMI medium. The rossetting of T lymphocytes with AET-treated SRBC was repeated once more in the exactly same way. If the cell pellet appeared red as a consequence of contamination with SRB cells 2 ml of the red cell lysis buffer was added (10 mM potassium bicarbonate, 155 mM ammonium chloride, 100 mM EDTA) and the cells incubated at 4 °C for 5 min. 50 ml of RPMI medium was added to wash the cells and after centrifugation at 12000 rpm for 5 min the cells were resuspended in 10 ml of the complete RPMI 1640 medium containing 10 % FBS and the antibiotics streptomycin, penicillin and gentamycin. The cells were counted and accordingly diluted to the final concentration of 1×10^6 cells/ml. For cell proliferation assay the cells were left in the medium for 24 h at 37 °C, in the atmosphere of 5 % CO_2.

In some experiments, following the two rounds of SRBC treatment the cells were applied on the top of discontinuous Percoll gradient which consisted of 30, 40, 45, 50, 55, 60 and 80 % Percoll in Hanks' balanced saline solution (HBSS) pH 7.2 and centrifuged at 28000 rpm /25 min, at 4 °C. The high density cells were collected at the interface between 55 and 60 % percoll, washed with RPMI 1640 medium and left overnight in the complete medium as described above.

2.4.8. Mitomycin treatment of cells

To eliminate the interference from non-B cells in the proliferation assays, the transformed fibroblast cells which were used as supplier of CD32 and CD23 were treated with mitomycin C, the inhibitor of DNA synthesis. This was usually done with 15-20 µg of mitomycin C per ml of medium for 12-14 h (overnight) or with 50 µg/ml for 2 h. The cells were then washed 3 times with PBS and incubated in the mitomycin free medium. The cells treated in this way can survive up to 3 days, which is sufficient time for the B cell proliferation assay.

2.4.9. B cell proliferation assay

The tonsillar B cells were used 24 h after the isolation and purification. The cells were seeded at the concentration of 2.5 x 10^5 cells/ml in complete RPMI medium with 10 % FBS and the appropriate stimuli of cell activation were added. The typical experiment was performed in 96-well plates in such a way that the resting B cells (5 x 10^4 cells/well in 0.2 ml medium) were co-incubated with the fibroblast cells (2 x 10^4 cells/well) expressing the low affinity receptor for IgG (CD32) in the presence of anti-IgM antibody DA4.4 and 200 units/ml of IL-4. Following 48 h incubation, 1 µCi of [*methyl*-^{3}H]-Thymidine was added to each well and the incubation continued for 12-16 h. The cells were harvested onto glass fib er paper and washed to remove free radioactivity prior measuring the counts. The amount of incorporated radioactive thymidine is proportional to the increase in DNA synthesis as the result of cell proliferation.

CHAPTER 3: EXPRESSION OF HUMAN SOLUBLE CD23 IN *ESCHERCHIA COLI*: BIOCHEMICAL AND BIOPHYSICAL PROPERTIES OF THE RECOMBINANT PROTEIN

3.1. Introduction

Naturally occurring human soluble CD23 has been reported to play several important biological roles, but, although some of these roles have been firmly established there is still no consensus about which soluble fragments display which biological activities and, in particular, little is known about the molecular structure and organization of soluble CD23. The later has become a very important aspect of studying the role of CD23, as there seems to exist a general agreement that knowledge of the relation between the structure and function of CD23 can be used to interpret a number of activities of this protein. Use of cell culture supernatants to study the function and the structure of soluble CD23 has proven to be not only difficult but can actually lead to controversial findings, with the main reasons being indeterminate composition of different fragments, insufficient amount of the protein and its instability. Therefore, a better approach to study the role and the structure of soluble CD23 is heterologous expression and purification of workable amounts of the protein. Several efforts in that direction have already been made. Sarfati *et al.*, 1992b, have reported the generation of a 16 kDa CD23 fragment in CHO cell line that was capable of suppressing IgE synthesis. Kalsner *et al.*, 1992, expressed soluble CD23 in both the yeast *Saccharomyces cerevisiae* and CHO cell line and found that the two products had different glycosylation. Graber *et al.*, 1992, reported expression and partial characterization of soluble CD23 in insect cells using baculovirus expression system. The authors showed that the recombinant protein was biologically active in at least two aspects: binding to IgE and promoting the survival of germinal centre B cells in synergy with interleukin 1. The insect cell expressed soluble CD23 was shown to be a monomer but the subsequent studies on this protein (Beavil *et al.*, 1995) showed that it forms dimers and trimers which can be chemically cross-linked.

It was one of the objectives of this study to generate a reliable source of soluble CD23 and study its structural and functional properties. The expression of proteins in *E. coli* has

several advantages over the other expression systems, in particular with respect to the amount of material obtained. No previous attempt of expression of CD23 in *E. coli* has been reported. Therefore, it was intended in this work to generate large amount of *E. coli* expressed CD23 and design optimal conditions for the recovery of its biological activity. Several considerations were taken into the account in making this choice of expression system. First, for the structural studies anticipated in this work sufficiently large amount of readily available material was required. Second, it was assumed that the bacterial product might be more stable than mammalian cell expressed protein, since the protease(s) that cleave(s) it is (are) unlikely to be present in the bacterial culture, as opposed to the animal cell cultures. And finally, another consideration taken into account was the fact that the bacterial cells are unable to glycosylate proteins and CD23, having one N-glycosylation and several potential O-glycosylation sites, produced in *E. coli* may represent a good model to study the role of glycosylation in animal type lectins. A parallel study (not part of this work) was undertaken with the aim of generating another recombinant form of soluble CD23 using a mammalian cell line, providing the possibility of comparison of the two recombinant forms of the protein from different sources and the study of the role of glycosylation, in particular.

However, it is well known from the literature that most of the proteins expressed in *E. coli* accumulate within the cell in the form of insoluble inclusion bodies (reviewed by Fischer *et al.*, 1992). To recover the protein, the insoluble protein pellets must be separated from the other cellular components and then solubilized in denaturants such as guanidine chloride or urea, followed by the chemical reduction of cysteines. The refolding of the protein *in vitro* is not always easy, but several different protocols are available and numerous proteins have been successfully refolded (Fischer *et al.*, 1992).

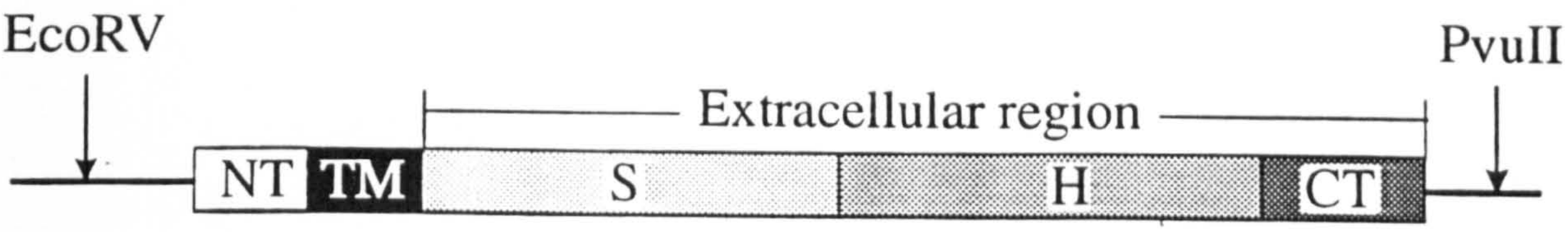
Of particular interest in this work was determination of the molecular weight and the oligomerisation state of soluble CD23 as well as the study of the biological activities of the recombinant protein with respect to binding of IgE and CR2. The experimental approach to these objectives was to design the optimal protocol for renaturation of the expressed protein and to test its binding properties. Several molecular weight determination methods including HPLC, native gel electrophoresis, sucrose gradient

centrifugation, dynamic light scattering and chemical crosslinking were used to distinguish between different oligomerisation forms of the protein.

3.2. Construction of the expression vector pRR28 and expression of CD23 in *E. coli*

The expression vector pRR28 was constructed by inserting the cDNA for the extracellular sequence of human CD23 into the plasmid pET-5a. The cDNA fragment was amplified by PCR and ligated into the plasmid Blue Script from which the *NdeI-BamHI* fragment was isolated and subcloned into the expression vector pET-5a. The plasmid contains the ampicillin resistance gene for amplification in bacteria and transcription of the inserted gene is driven by the bacteriophage T7 promoter which is controlled by IPTG inducible *lac* promoter/operator. The T7 RNA polymerase is supplied by the host strain BL21/DE3 and the induction is achieved with 0.4 mM IPTG in minimal medium M9. The expressed CD23 polypeptide starts with the aspartic acid residue at position 48 and includes the whole stalk region of the protein, the lectin domain and the C-terminal sequence. A schematic representation of the domains of CD23 together with the expression vector pRR28 is shown in Fig.3-1. The expression of CD23 by the transformed strain BL21 was tested in a small scale culture of 5 ml. The rapidly growing cells were divided in ratio 1:20 and incubated long enough to reach the OD 0.6-1.0, after which 0.4 mM IPTG was added and the cells incubated a further 6-10 h. To confirm the expression of CD23 and also to screen the transformants for the level of expression, the cells from 5 ml culture of different clones were harvested and lysed directly in 100 µl of SDS-PAGE sample buffer and a 20 µl aliquot was applied on a 15 % gel together with the negative control (non-induced cells). The Coomassie staining of the gel showed the presence of a dominant protein band with the apparent molecular weight of 35 kDa which was not present in the negative control (Fig.3-2). No significant difference in the level of expression among the tested transformants could be observed. This protein band was subsequently shown to be immuno-reactive with a polyclonal antibody to CD23 (Rb55) while no immuno-reactive material could be seen in the negative control (Fig.3-3).

(a)



(b)

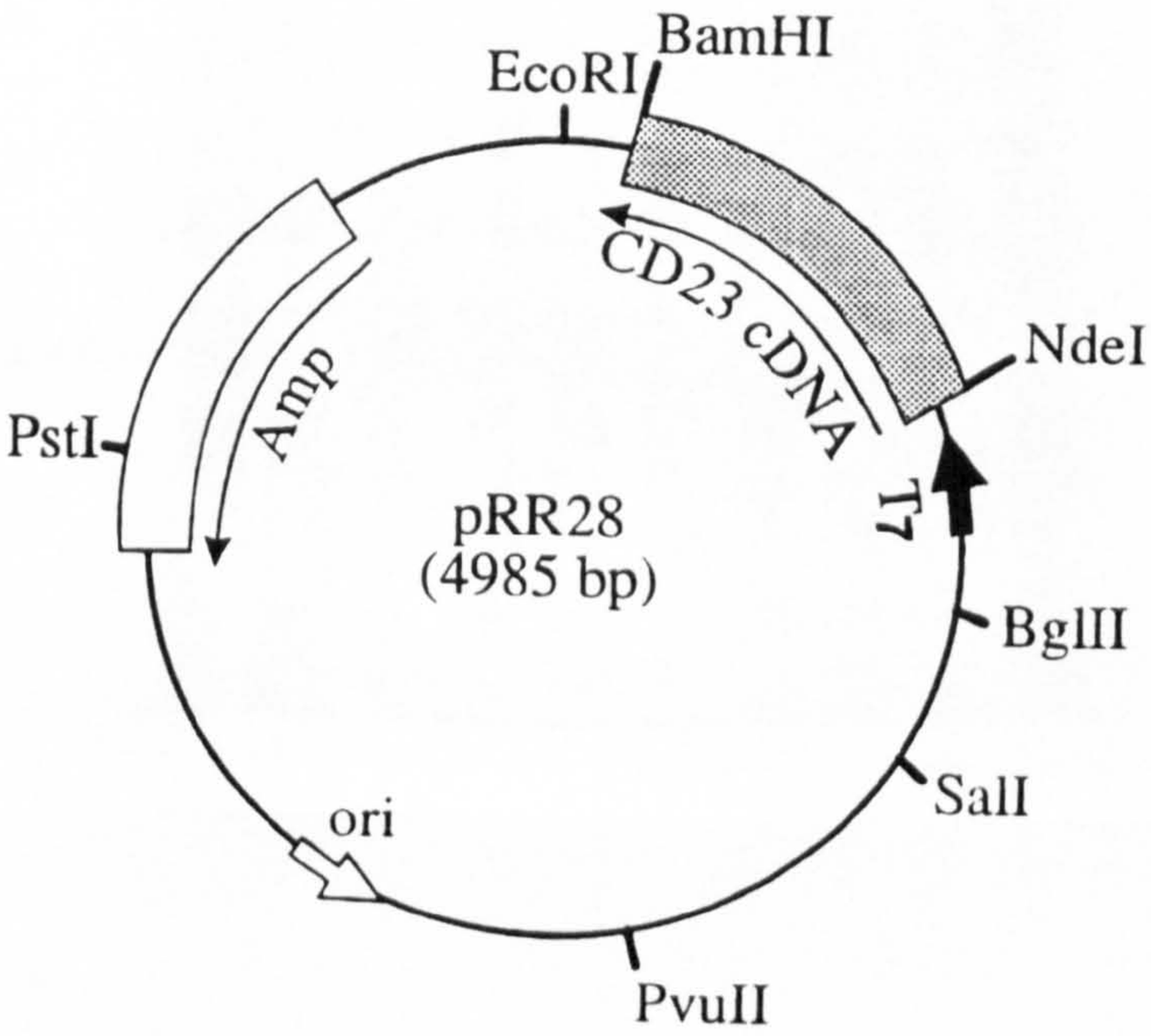


Fig.3-1 Expression vector pRR28 for expression of human CD23 extracellular sequence in *E. coli.*
Above is shown the linear map of human CD23 cDNA and the discrete protein domains of CD23 protein are marked (a); NT, N-terminal sequence, TM, transmembrane region, S, stalk, H, head domain, CT, C-terminal sequence. b) The expression vector pRR28 contains the extracellular CD23 cDNA fragment as indicated on the linear map; the plasmid contains IPTG inducible T7 promoter and the ampicillin resistance gene.

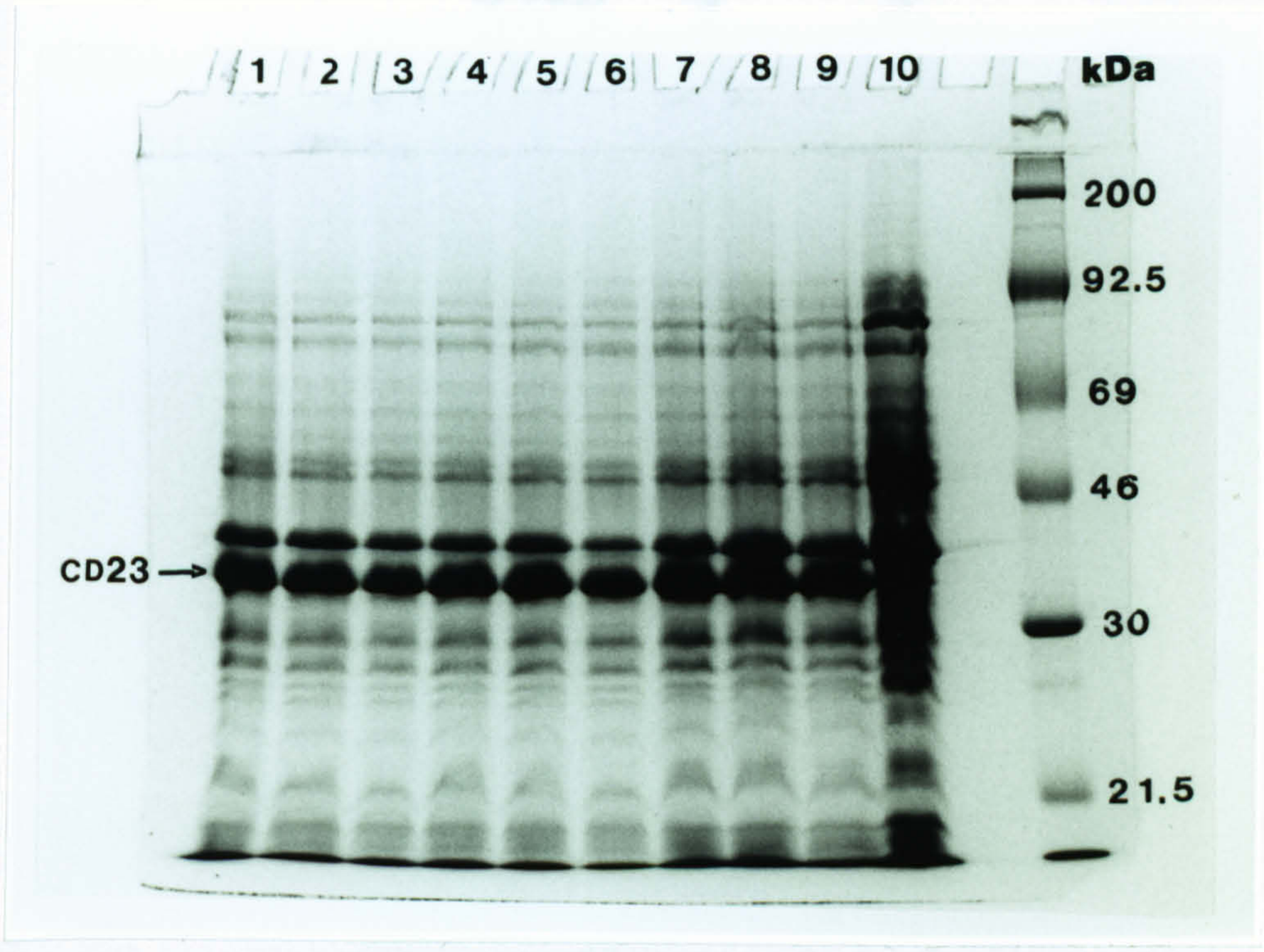


Fig.3-2 Expression of CD23 in *E. coli.*
The bacterial culture (strain BL21) was grown until OD_{600} reached 0.6-1.0 and 0.4 mM IPTG was added. Following 10 h incubation the cells were harvested, lysed in SDS-PAGE sample reducing buffer and analyzed by electrophoresis and Coomassie staining. Lanes 1-9 represent different clones of transformed cells; lane 10 is the negative control (cell lysate from non-transformed cells). CD23 is represented by the massive protein band (indicated by the arrow) of an apparent molecular weight of 35000. No significant difference in the level of expression between the tested clones was observed.

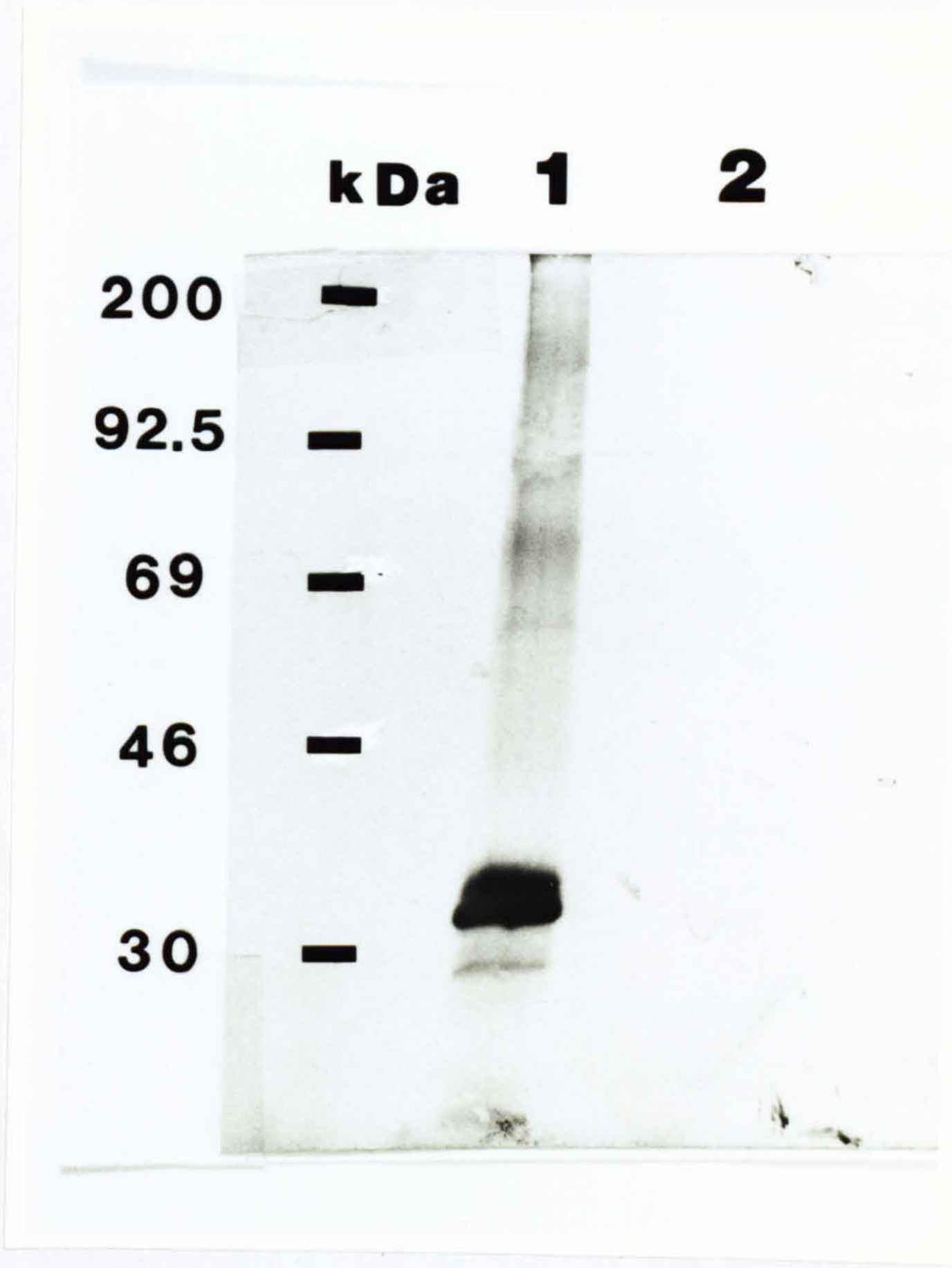


Fig.3-3 Western blot of recombinant CD23.
A sample of cell lysate from transformed (lane 1) or nontransformed cells (lane 2) was treated with 10 mM DTT and applied on SDS-PAGE followed by transfer of separated proteins to nitrocellulose filter. The polyclonal anti-CD23 antibody Rb55 was used to detect CD23 in the cell lysate. The result confirms that the 35 kDa protein from the previous figure is indeed CD23.

3.3. Extraction and purification of recombinant CD23 from bacterial pellet

The recombinant CD23 generated by *E. coli* is expressed in insoluble and biologically inactive form, as inclusion bodies. To obtain the soluble protein the insoluble inclusion body material was separated from other cell components by cell disruption, homogenisation by several rounds of sonication and washing in buffers containing non-ionic detergent Nonidet NP-40, and finally solubilised in 6 M GnCl. DTT was added to complete the reduction of the present sulphydryl groups and CD23 purified by gel filtration on a Sephacryl S-100 column with 6 M GnCl as the running buffer (Fig.3-4). The fractions were analyzed for CD23 by electrophoresis and Western blotting, and the positive fractions were pooled together. This was concentrated to 1 mg/ml by ultrafiltration (Amicon) and kept at 4 °C as the guanidine stock of CD23. The typical yield of the purified protein from 1 l bacterial culture was 50 mg which compares favourably with other expression systems.

3.4. Refolding of recombinant CD23

3.4.1. Partial refolding of CD23 by the method of dilution

In order to test preliminarily the ability of the recombinant CD23 to fold correctly, an experiment was designed in which the concentration of the denaturant (GnCl) was reduced stepwise to 2, 1, 0.6 and 0.2 M, respectively, by dilution in 0.1 M Tris buffer pH 7.8. Each sample was then analyzed by measuring CD (circular dichroism) spectrum. Fig.3-5 shows the increase of α-helical structure in the partially refolded protein with the decrease of concentration of GnCl.

3.4.2. Refolding of CD23 by the method of dilution and dialysis

A protein refolding protocol was applied in which guanidine stock of CD23 was diluted to 50 µg/ml in 0.1 M Tris buffer, pH 7.8 (guanidine concentration was 0.3 M), and incubated for 12 h at 4 °C, followed by the further dilution of the protein to 10 µg/ml in redox buffer which consisted of 25 mM Tris, pH 7.8, 1.0 M NaCl, 25 mM $CaCl_2$, 0.1 mM

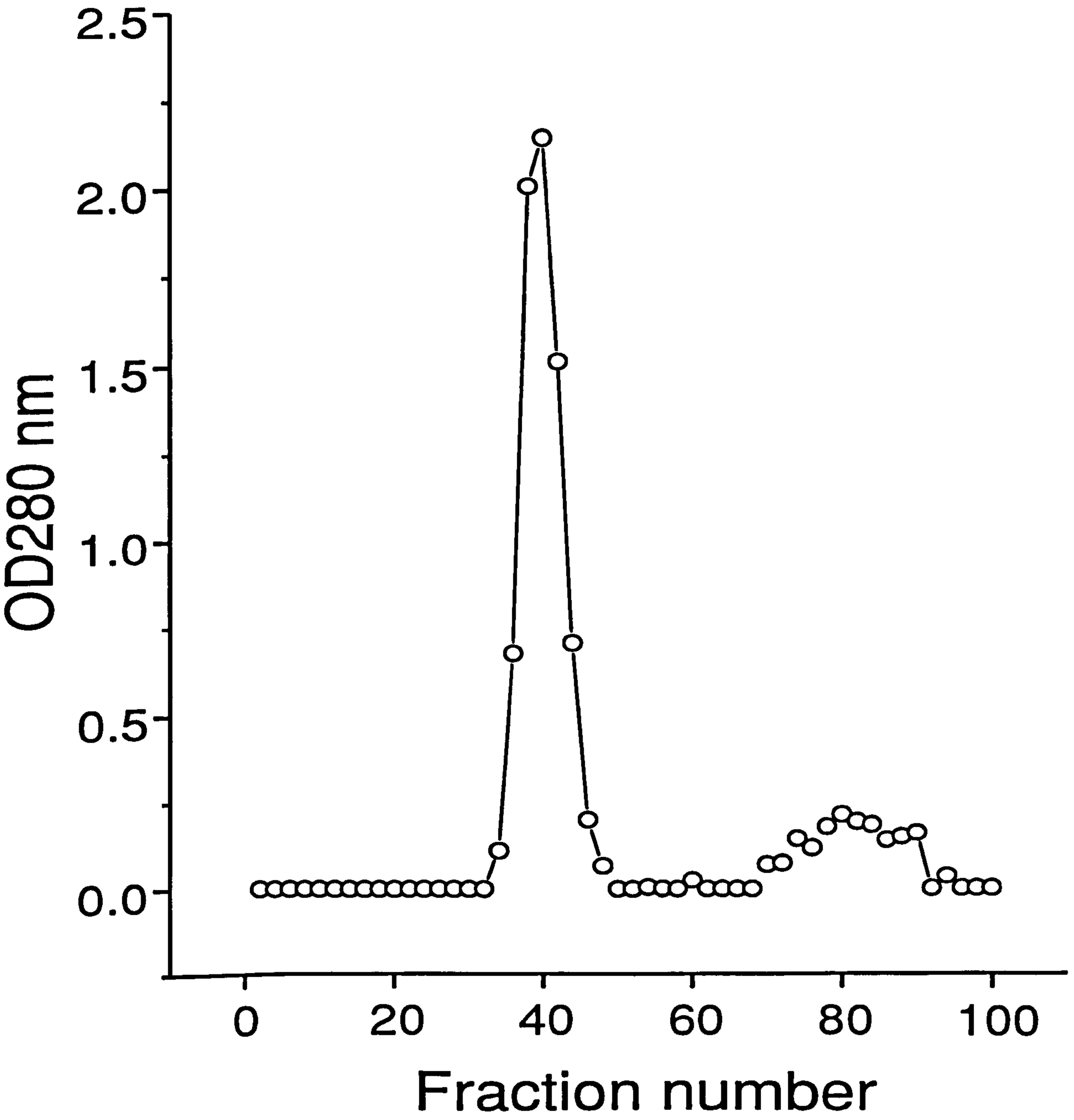


Fig.3-4 Gel filtration of denatured recombinant CD23 on Sephacryl S-100 column. The GnCl solubilised CD23 preparation following the extraction from bacterial pellets was incubated with 10 mM DTT and applied on a Sephacryl S-100 column pre-equilibrated with 6 M GnCl and gel filtration performed as described in Chapter 2. The fractions were analyzed for protein content (OD_{280} nm) and for CD23 by SDS-PAGE and Coomassie staining. Above is the gel filtration profile obtained by spectrophotometric analysis of fractions. The large peak represents CD23 the purity of which was subsequently estimated at 95 %.

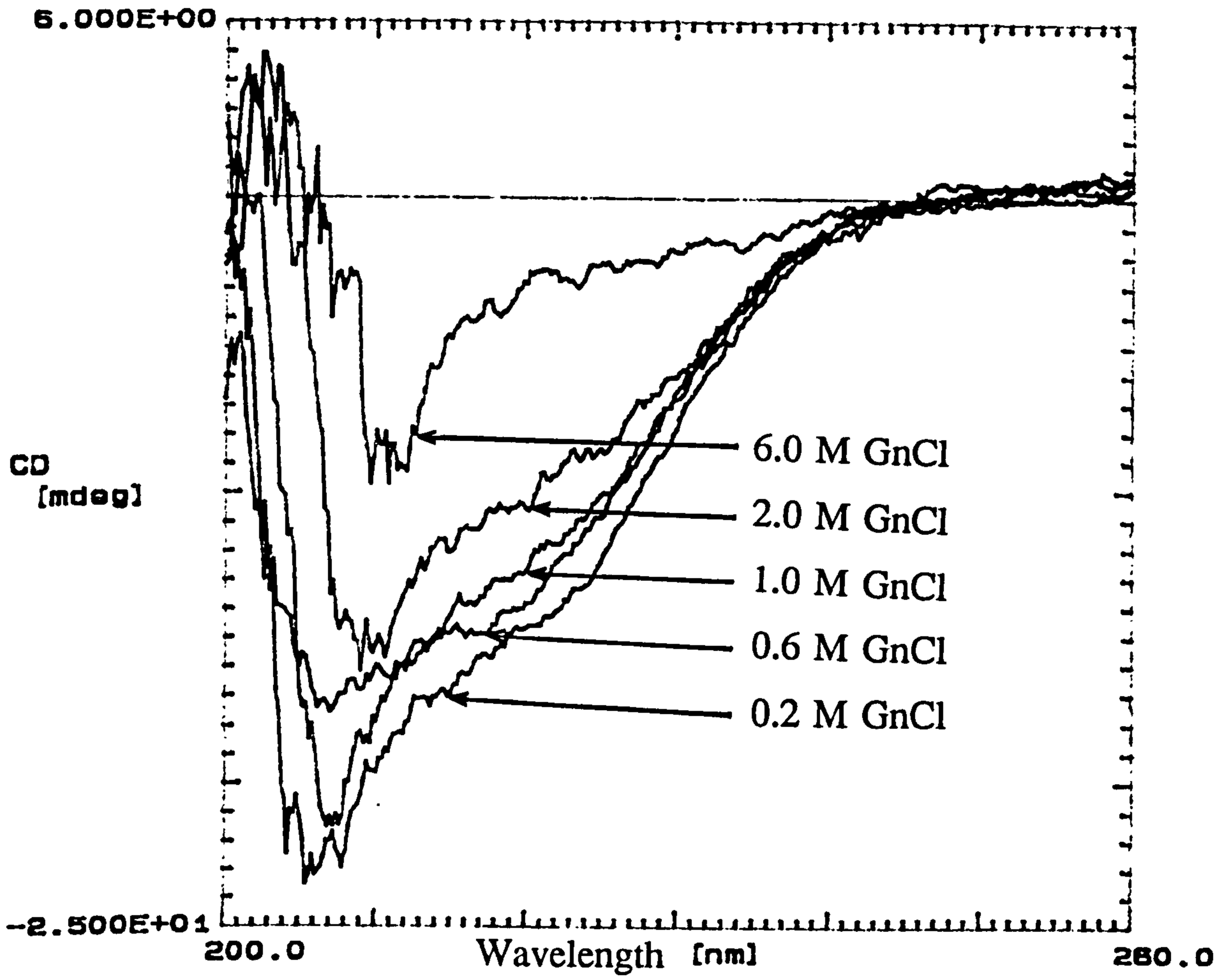


Fig.3-5 Circular dichroism (CD) spectra of partially refolded CD23 by dilution in renaturation buffer.

The shown CD23 CD spectra were obtained in the presence of different concentrations of GnCl covering the range 6.0-0.2 M (baseline subtracted). The renaturation buffer used was 0.1 M Tris, pH 7.8. The increase in size of the minimum peaks at 205 and 220 nm is characteristic of α-helical secondary structure formation. The minimal concentration of GnCl that still allows significant structure formation was 1.0 M.

oxidized glutathione, 0.01 mM reduced glutathione and 0.6 M GnCl. The sample was dialysed against 20 volumes of the same buffer for 24 h and against 20 volumes of 100 mM Tris, pH 7.8, 2 mM $CaCl_2$ for the same time period. The protein was concentrated to 1 mg/ml by ultrafiltration and kept frozen in aliquots at -20 °C. This refolding protocol was designed to favour an early oxidation of the sulphydryl groups followed by a more limited degree of freedom formation of secondary structure in partially refolded protein. A good indicator of folding efficiency, in particular with respect to correct intramolecular pairing of sulphydryl groups,is the percentage of the aggregated protein in the preparation after the completion of the refolding protocol. This can be checked by SDS-PAGE, by running parallel non-reduced as well as the reduced sample of the protein. As can be seen in Fig.3-6 this refolding protocol resulted in formation of nonspecific protein aggregates while only a minor proportion of the protein remained in soluble form. The recovery of the protein cannot be considered satisfactory and, therefore, other refolding protocols were tested.

3.4.3. Refolding of CD23 by the method of mixed disulphides

This protein refolding protocol was previously successfully applied for renaturation of the protease papain by Taylor *et al.*, 1992, and after testing a number of other refolding protocols (including the one described above) was found in this work to be the optimal protocol for refolding of recombinant CD23. The protocol, as described in details in "Materials and methods" utilizes blocking of the 8 sulphydryl groups of CD23 by conversion to mixed disulphides with high concentration of oxidized glutathione. The initiation of correct pairing of cysteine residues is achieved by addition of free cysteine to displace glutathione from the protein-S-S-glutathione mix and further dilution of the sample to prevent intermolecular interactions. The oligomerisation of the protein was then induced by concentrating the sample to a final concentration 1 mg/ml. The SDS-PAGE showed that the CD23 preparation had low percentage of the aggregated form and that most of the protein remained intact (Fig.3-7, lanes 3 and 4). However, it is worth noticing that the presence of protease inhibitor TLCK is crucial for the stability of CD23, as indicated by significant decomposition of the protein in the absence of the inhibitor (lanes 1 and 2 in Fig.3-7).

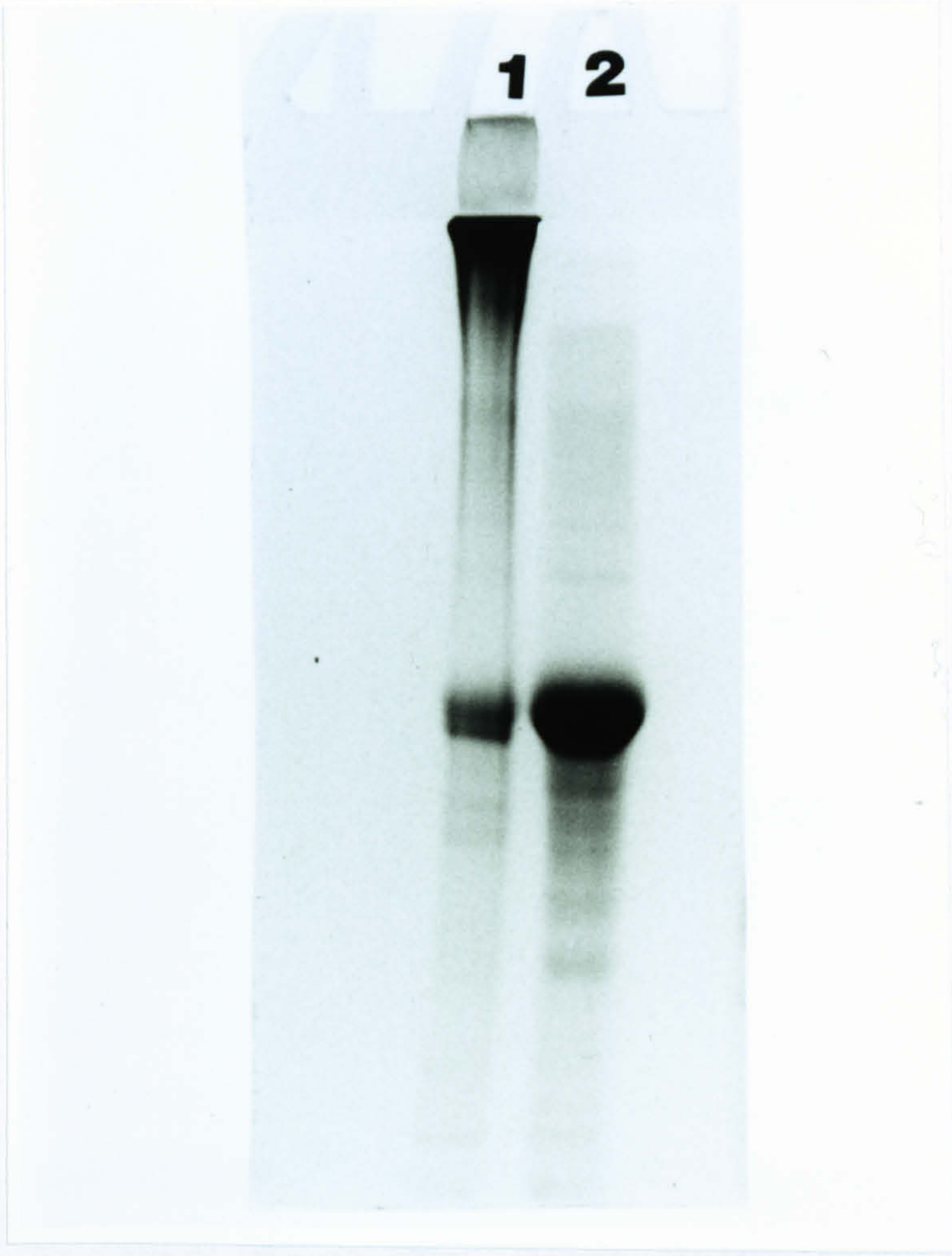


Fig.3-6 Refolding of CD23 by method of dilution and dialysis.
The figure shows an unsuccessful attempt to refold CD23 by direct dilution in renaturation buffer and dialysis against the same to remove the denaturant. Coomassie stained gel shown above is SDS-PAGE analysis of the refolded material (20 µg) in the absence (lane 1) and presence (lane 2) of reducing reagent (10 mM DTT). Most of the protein is in the aggregated form indicating that this refolding protocol is not appropriate for renaturation of CD23.

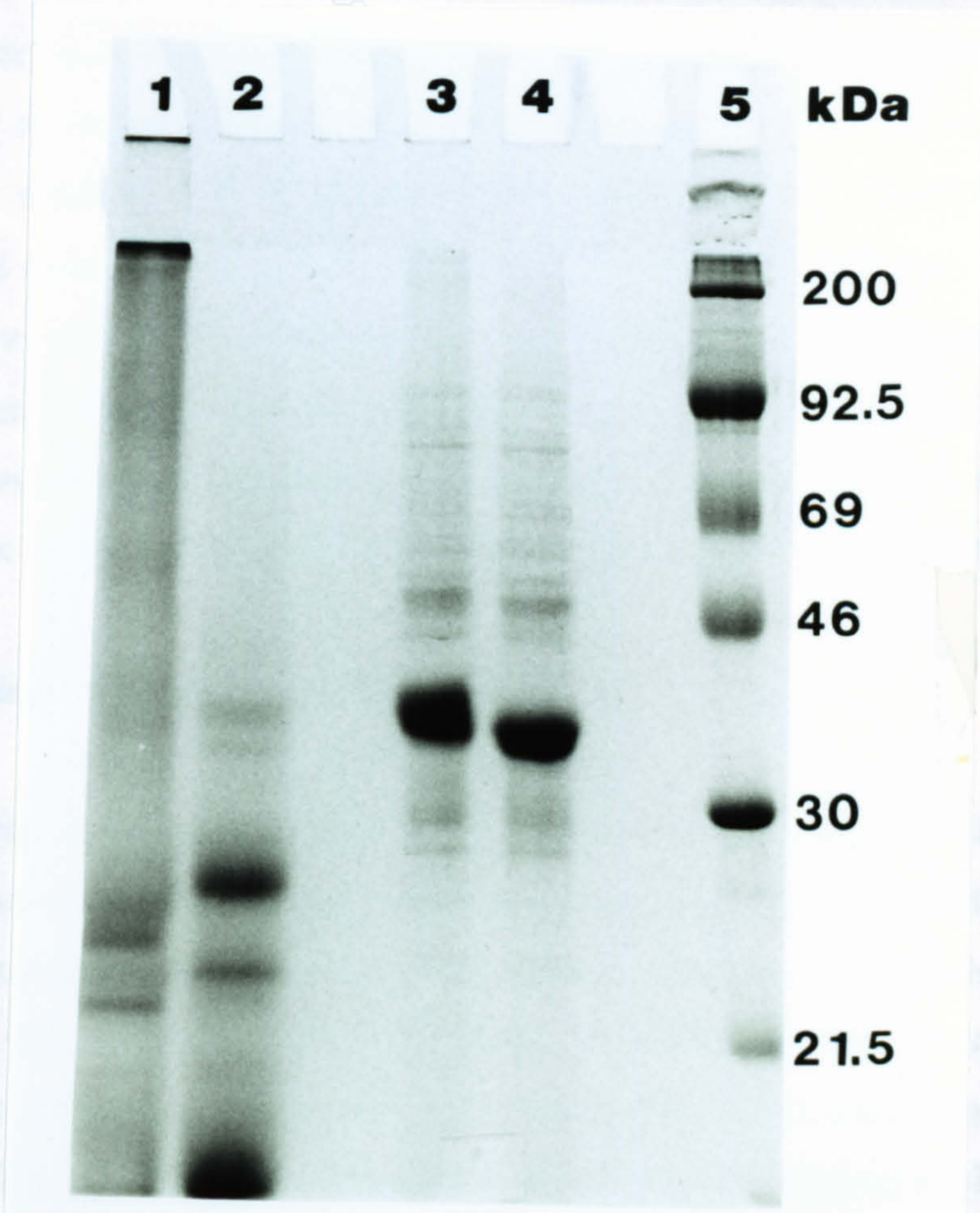


Fig.3-7 Refolding of CD23 by method of mixed disulphides.
In contrast to the previous protocol (Fig.3-6) the protein obtained by this refolding technique was mainly in soluble form as can be seen from the SDS-PAGE analysis of the protein sample (20 µg) in nonreducing or reducing conditions (lanes 3 and 4). The lanes 1 and 2 is the same except that the protease inhibitor TLCK was not added at the end of refolding protocol; most of the protein was degraded, thus demonstrating the requirement of this inhibitor for preservation of intact CD23 structure from (auto)proteolytic cleavage. Lane 5 is high molecular weight protein markers from Amersham.

3.4.4. CD spectrum of refolded recombinant CD23

The CD spectrum of refolded recombinant CD23 was measured in Tris buffer pH 8.0 at the protein concentration of 400 µg/ml. The spectrum obtained (Fig.3-8) indicates a minimum at 205 nm typical of α-helical structure but the calculated ratios of each particular structure (15 % α-helix, 56 % β-sheet, 29 % random) fall short of the theoretical estimates for α-helical structure (50 %) on the basis of molecular modelling (Beavil *et al.*, 1992). One possible explanation for this could be a significant underestimate of α-helical structure by the employed programme but it is likelier that the soluble form of the protein may have less α-helical structure than the membrane form due to the lack of hydrophobic transmembrane domain which could stabilize the α-helix.

3.5. Binding of recombinant CD23 to IgE

The capacity of the recombinant CD23 to bind IgE was tested by ELISA immunoassay and plasmon surface resonance (SPR) assay. In the ELISA assay, CD23 was coupled to the plate and IgE added as the second layer. After incubation and washing, the plates were incubated with anti-IgE antibody followed by incubation with the secondary antibody-peroxidase complex and detection with chromogenic peroxidase substrate. The result is shown in Fig.3-9. As can be seen on the graph, the recombinant CD23 binds to IgE in a concentration dependent manner. This result was confirmed by SPR, a similar but rather more direct binding assay. In this case CD23 was coupled to the surface of a sensor chip and IgE-Fc was added at 4 different concentrations: 2, 1, 0.5 and 0.25 µM. The association (k_{+1}) and dissociation (k_{-1}) rates were measured and the affinity constant (Ka) was calculated as k_{+1}/k_{-1}. The calculated Ka constant was 2.1×10^6 M^{-1}. The obtained sensograms for binding of recombinant CD23 to IgE-Fc are shown in Figure 3-10 and the calculated values for the kinetic parameters and Ka constant are shown in Table 3-1.

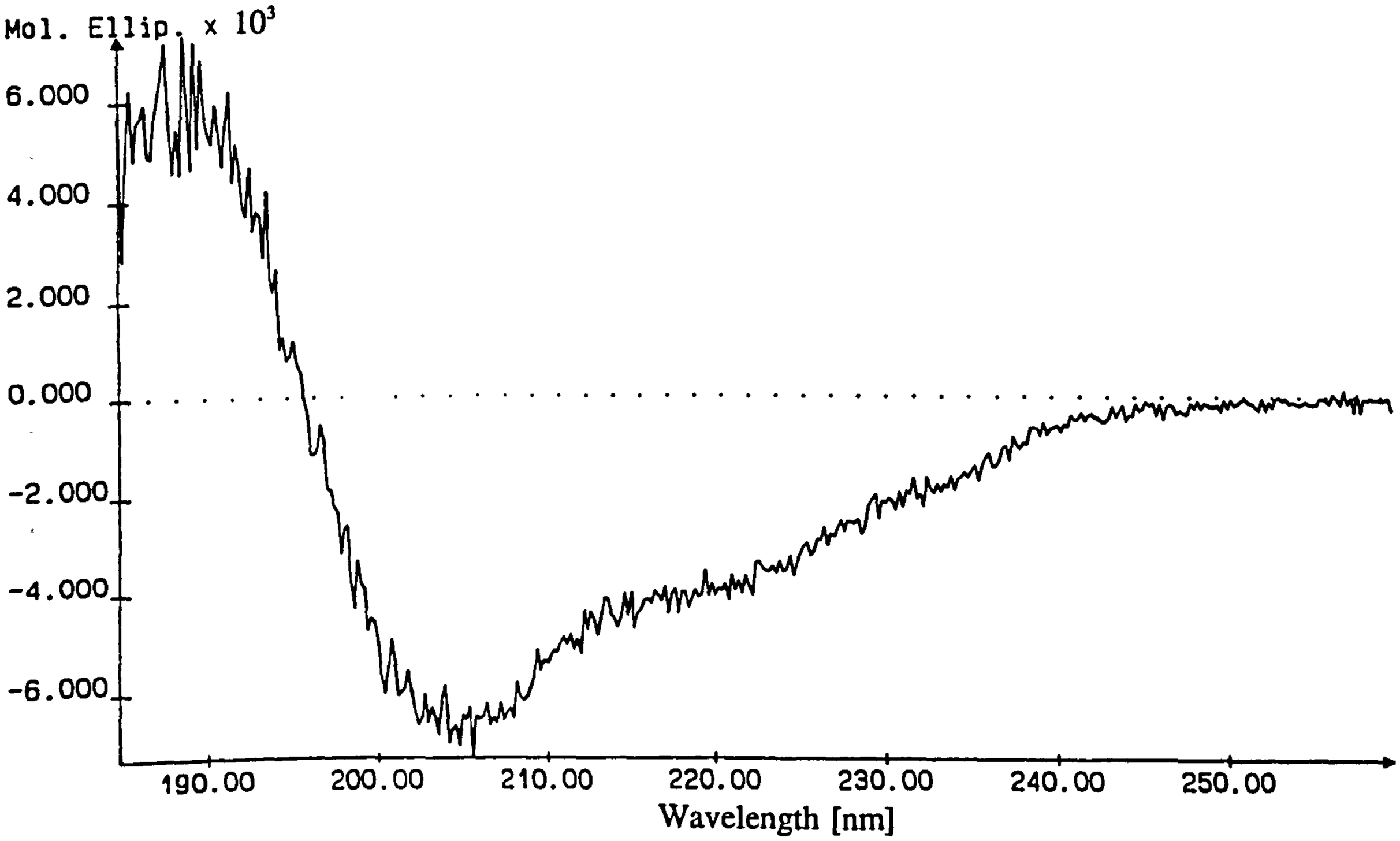


Fig.3-8 CD spectrum of refolded recombinant CD23.
The spectrum was taken from the final preparation of the refolded CD23 at 0.4 mg/ml; the baseline is subtracted. The estimated amount of α-helical structure was 15 %; 56 % was β-sheet and 29 % random coil. The molecular elipticity (ordinate) is expressed in mdeg x cm^2/dmol.

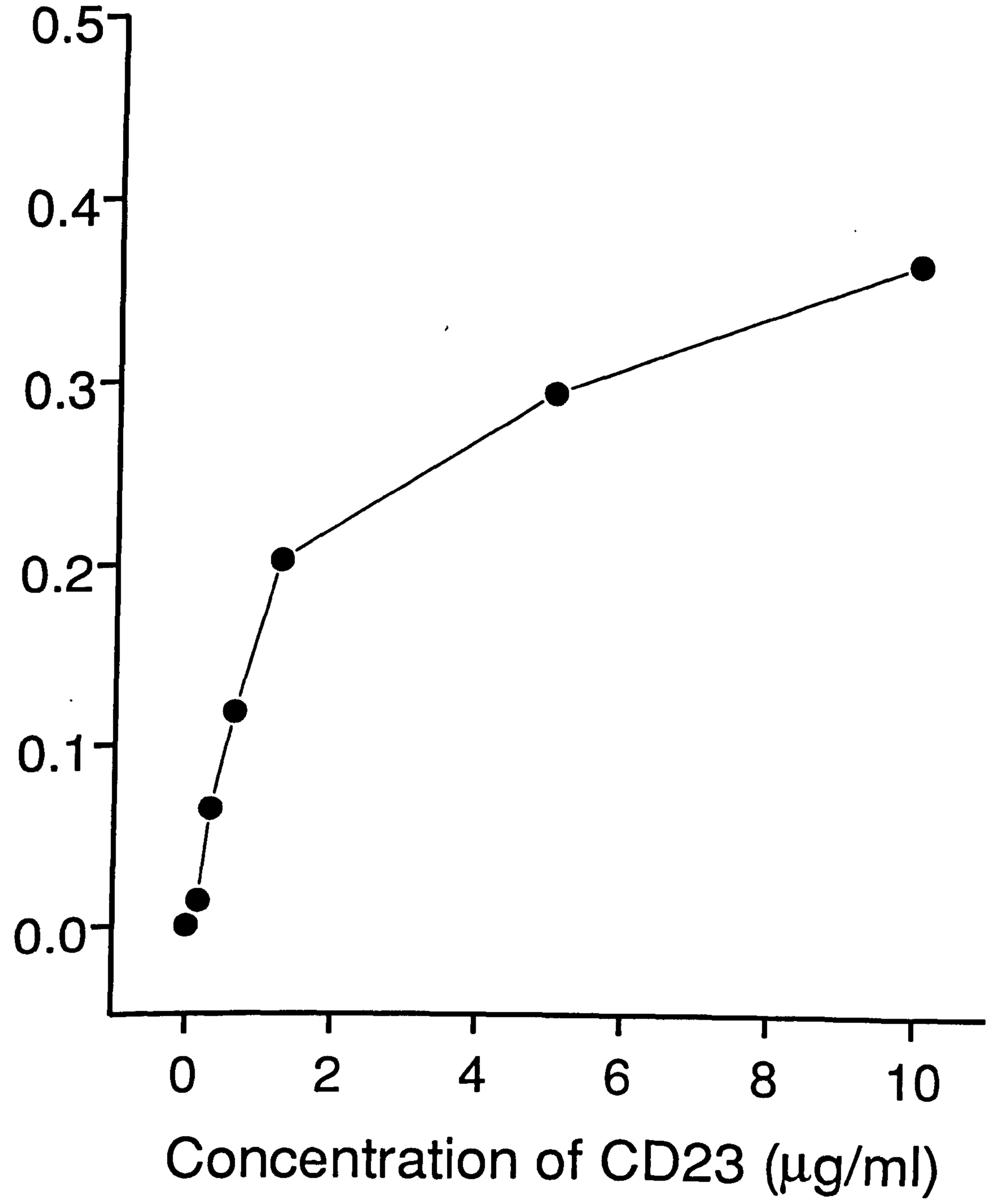


Fig.3-9 Binding of recombinant CD23 to IgE in ELISA assay.
The capacity of recombinant CD23 to bind IgE was tested by ELISA assay in which CD23 was coupled to the plate and IgE added in solution, followed by addition of anti-IgE and secondary antibody peroxidase conjugate. The intensity of the developed yellow colour in the peroxidase reaction was measured at 450 nm; the negative control (no IgE added) has been subtracted.

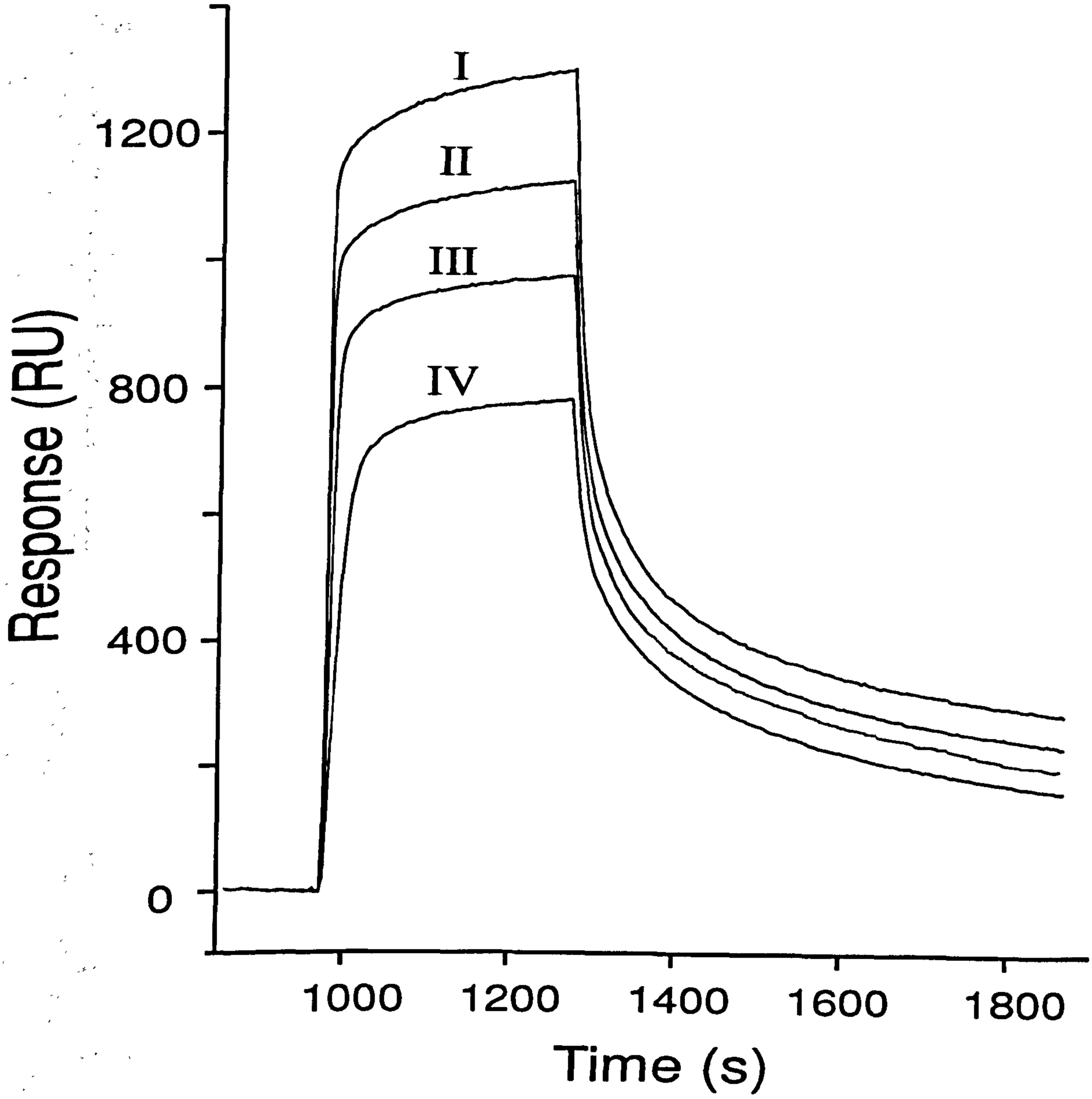


Fig.3-10 Binding of recombinant CD23 to IgE analyzed by SPR assay.
CD23 was immobilised on BIAcore sensor chip and IgE-Fc was allowed to bind for 5 min after which period the dissociation was induced by the free flow of the binding buffer (TBS pH 7.5, 2 mM $CaCl_2$); the different curves represent the sensograms obtained with the following concentrations of IgE-Fc (in μM): 2 (I), 1 (II), 0.5 (III) and 0.25 (IV), respectively.

Table 3-1: Measurement of the affinity constant for recombinant CD23 binding to IgE.

Conc. of IgE-Fc (M)	k_{+1} x 10^3 ($M^{-1}s^{-1}$)	k_{-1} x 10^{-3} (s^{-1})	Ka x 10^6 (M^{-1})
2 x 10^{-6}	5.7	4.5	1.3
1 x 10^{-6}	5.4	4.8	1.1
5 x 10^{-7}	10.6	4.5	2.4
2.5 x 10^{-7}	17.4	4.8	3.6
The average affinity constant for CD23 interaction with IgE-Fc: 2.1 x 10^6 M^{-1}			

3.6. Determination of oligomerisation state and molecular weight of recombinant CD23

3.6.1. Native PAGE

In order to measure the molecular weight of recombinant CD23 under native conditions, a sample of the protein was run on a gradient gel (5-20 %) in the absence of SDS and DTT. Since the migration of proteins in native electrophoresis gels is dependent on both the size of the protein and the total charge of free residues it is impossible to measure the exact molecular weights of proteins as in the case of SDS-PAGE. Nevertheless, this can be compensated to some extent by overrunning the gel and getting the proteins to decrease the migration rate in the part of gradient where the concentration of polyacrylamide is sufficiently high to prevent further migration. The consequence of this is that the contribution of the charge of the protein to the migration rate and resolution becomes smaller while contribution of the size of protein increases; in this way one can estimate the molecular weight of the protein within reasonable limits of the experiment. In the case of CD23, the molecular weight estimate is 70000-100000, based on the comparison with BSA monomer (66000) and BSA dimer (132000). Fig.3-11 shows a native 5-20 % polyacrylamide gradient gel run for 4 h and stained with Coomassie blue , in which CD23

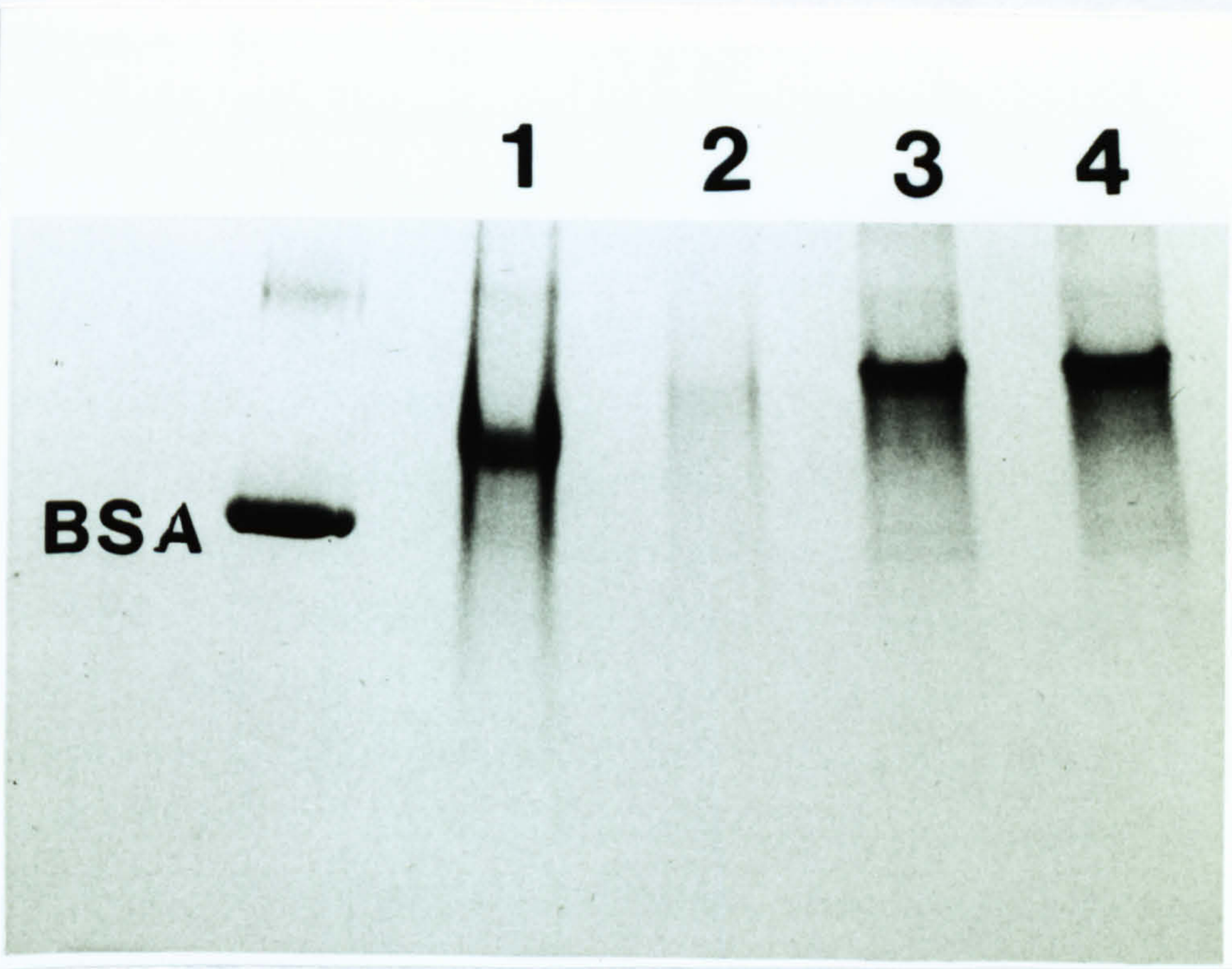


Fig.3-11 Native PAGE of recombinant CD23.
The sample of CD23 (20 µg) was applied on native gel with linear gradient of polyacrylamide concentration 5-20 % and the electrophoresis performed as described in Chapter 2. Lanes 1 and 2 represent denatured samples (either by 8.0 M urea, lane 1, or by 8.0 M urea plus 5 min boiling, lane 2); this was aimed to be internal marker control for CD23 monomer, but was obviously not sufficient to keep the protein denatured (1) or denatured and in solution (2). Lanes 3 and 4 are identical samples of CD23 in the absence of any denaturing reagents. The molecular weight of the two internal molecular weight protein markers (BSA monomer and dimer) are 66000 and 132000; under the experimental conditions applied CD23 migrates as a protein of the molecular weight between 66000 and 132000 (see also Fig.6-1, Chapter 6).

is represented by a diffuse protein band of the indicated molecular weight.

3.6.2. Chemical crosslinking of CD23 with EDC

For the determination of the oligomeric state of recombinant CD23 one of the experimental approaches in this work was chemical cross-linking of the protein subunits with the crosslinker EDC. This particular crosslinker was chosen because of its property to crosslink only the amino and carboxyl groups which are sufficiently close to each other or ideally part of the ionic bridges that can be formed between lysine and glutamic or aspartic acid residues, for example. The ionic bridges participate in stabilization of the α-helical stalks of CD23 through which the oligomerisation of the protein is thought to occur (Beavil *et. al.*, 1992). Several parameters which influence the efficiency of crosslinking were tested, including the ratio EDC/protein, pH of the buffer and the time required for the completion of the reaction. A typical reaction was carried out in 20 µl reaction volume which contained the appropriate volume of protein preparation, 2 µl of 250 mM (10X) sodium acetate buffer, 2 µl EDC 10X solution and the rest was distilled water. The influence of pH of the buffer on the crosslinking efficiency was tested over the pH range 3.0 - 6.0 and it was found that the optimal pH of the reaction was 5.0-5.5 (Fig.3-12). All the subsequent reactions were carried out at the pH 5.0 and at room temperature. The final concentration of the crosslinker in the reaction was typically 25 mM unless stated otherwise.

The crosslinking of recombinant CD23 was carried out at three different concentrations of the protein: 2, 50 and 500 µg/ml, using three different detection methods: autoradiography, silver and Coomassie staining. For the lowest concentration of CD23, an iodinated sample was used, detected by autoradiography. Fig.3-13 shows an increase in the yield of the dimeric form with the increasing concentration of the crosslinker and the appearance of trimer at the highest concentration of EDC. Further increase of the concentration of EDC did not change the ratio of dimer to trimer, neither did the prolonged incubation time (2 h, not shown). In order to find out if the ratio dimer/trimer is concentration dependent, that is, if the trimer is more easily formed at higher protein concentrations, an experiment was designed in which constant concentration of labelled

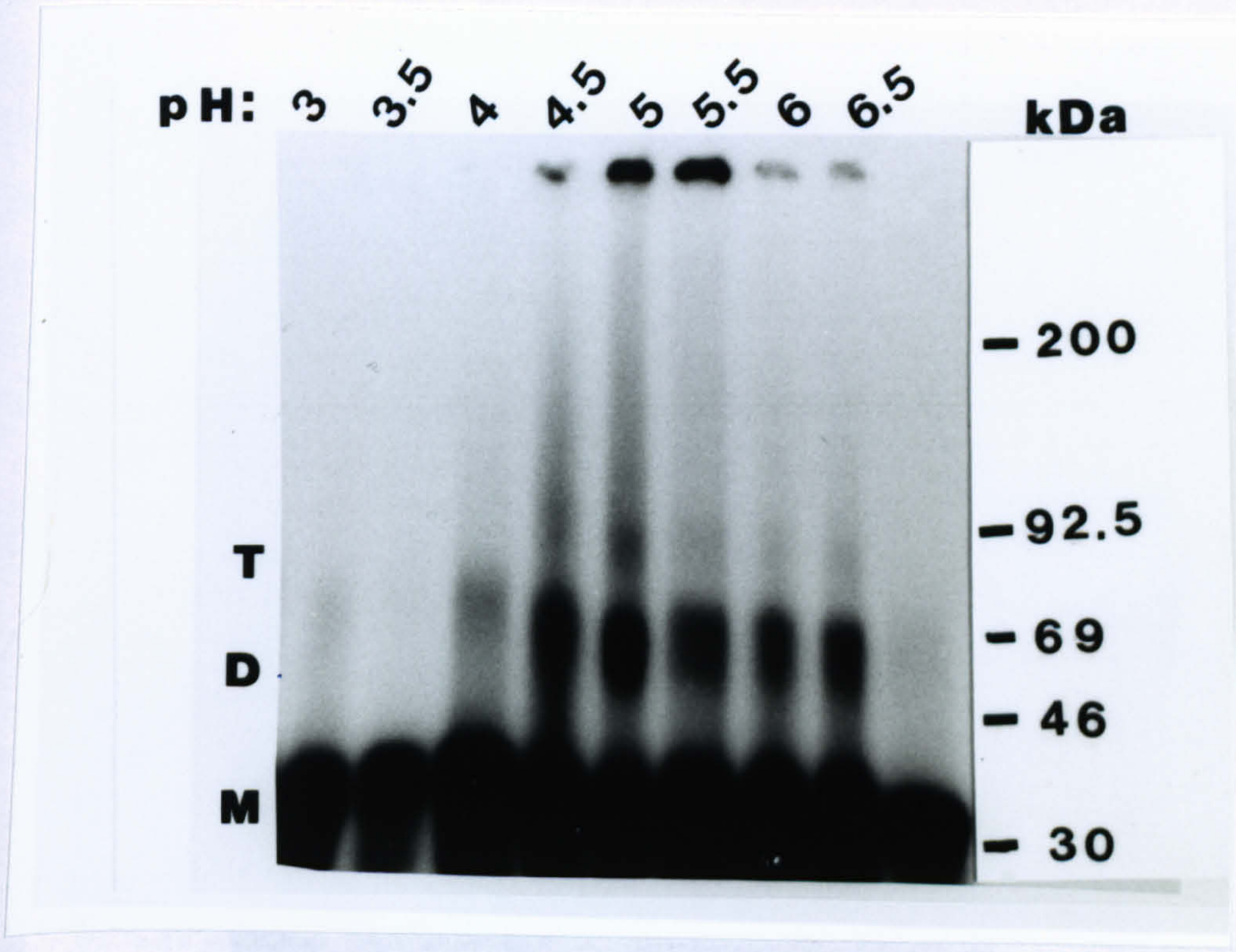


Fig.3-12 Optimal pH for crosslinking of CD23 with EDC.
The effect of pH of the buffer on efficiency of crosslinking was studied; the buffer solutions (sodium acetate, final concentration 25 mM) in the pH range 3.0-6.5 were prepared and added to the reaction mixture together with a constant concentration of the crosslinker and [^{125}I]-labelled protein. Following SDS-PAGE in reducing conditions the gel was dried and autoradiography was performed. The optimal pH of the buffer for crosslinking of CD23 with EDC was found to be 5.0. The letters T, D and M in the above picture mark the positions of CD23 trimer, dimer and monomer, respectively.

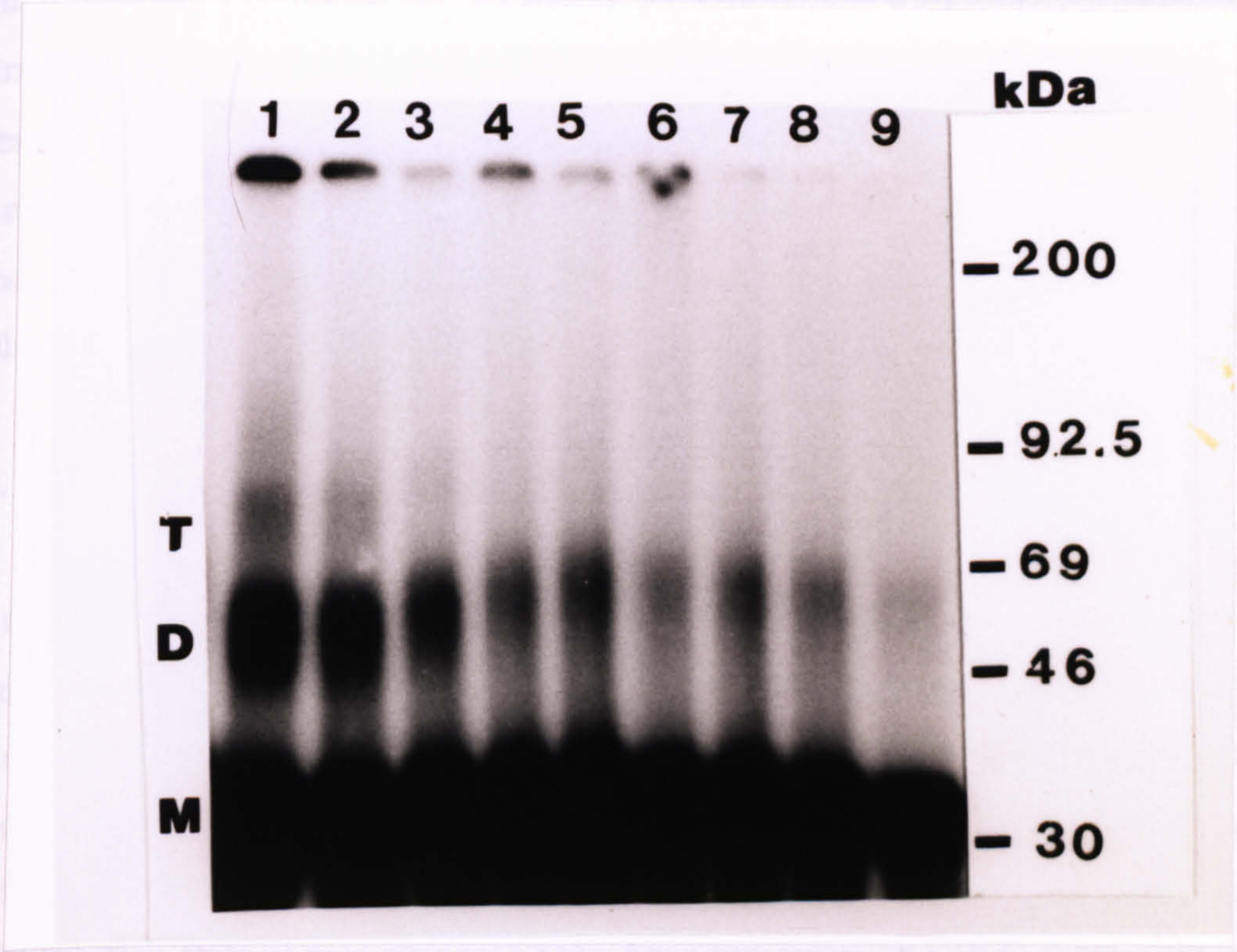


Fig.3-13 The effect of the ratio protein/crosslinker on the crosslinking of CD23. The concentration of the [^{125}I]-labelled protein in the reaction mixture was 2 µg/ml and the concentration of EDC was in the range 0.01-100 mM (from lane 8 to 1: 0.01, 0.1, 1, 5, 10, 25, 50 and 100 mM; lane 9 is negative control (no crosslinker added)). Under the experimental conditions no further increase of efficiency of crosslinking could be achieved with concentrations of EDC above 100 mM (not shown). The letters T, D, and M mark the positions of CD23 trimer, dimer and monomer, respectively.

CD23 was used and increasing amounts of the unlabelled protein added. The consequence of this should be that the yield of the formed trimer should increase with the increasing concentration of the unlabelled protein for as long as the concentration of EDC is not the limiting factor. Indeed, as can be seen in Fig.3-14 the increase of concentration of added unlabelled CD23 results in the increase of the yield of trimer as compared with the labelled protein alone, but after the unlabelled protein exceeded a critical concentration at which it actually competes for binding of the crosslinker with labelled CD23, the yield of trimer began to decrease. To confirm that this was true, CD23 was crosslinked at the concentration of 50 and 500 µg/ml, respectively. In both cases (Fig.3-15 and 3-16) the dominant form of the crosslinked CD23 was the trimer. As expected, with the increase of the concentration of the crosslinker some of the protein was intermolecularly crosslinked, resulting in the formation of nonspecific aggregates.

3.6.3. HPLC analysis of recombinant CD23

The fact that recombinant soluble CD23 can form trimers that can be detected by chemical crosslinking may not be definitive proof that this is the actual oligomerisation state of CD23 or the predominant form of the protein because of the possibility that there may exist an equilibrium between the protein monomer and trimer, with the dimer as an intermediate form. Other experiments were therefore carried out to investigate the oligomerisation state of recombinant CD23. On a Superdex-75 HPLC column (Pharmacia, optimal separation range 5000-70000) CD23 gives the profile shown in Fig.3-17. The larger of the two major peaks corresponds to a 65 kDa protein while the smaller peak corresponds to a 33 kDa protein. These molecular weights which were calculated from the standard curve obtained with the proteins of known molecular weights clearly correspond to the dimer and monomer of CD23. Although it is possible to notice the presence of the third small peak which might represent the trimer, it is impossible from this HPLC column, which has the size exclusion limit 75000, to conclude whether this might indeed be true; in addition to that, the proportion of this protein fraction in the total preparation is rather low (approximately 20 %). The ratio dimer/monomer does, however, increase with the increase of the concentration of the protein applied on the column (Fig.3-17c and d). The native CD23 showed a profile with one major peak which comes in the void

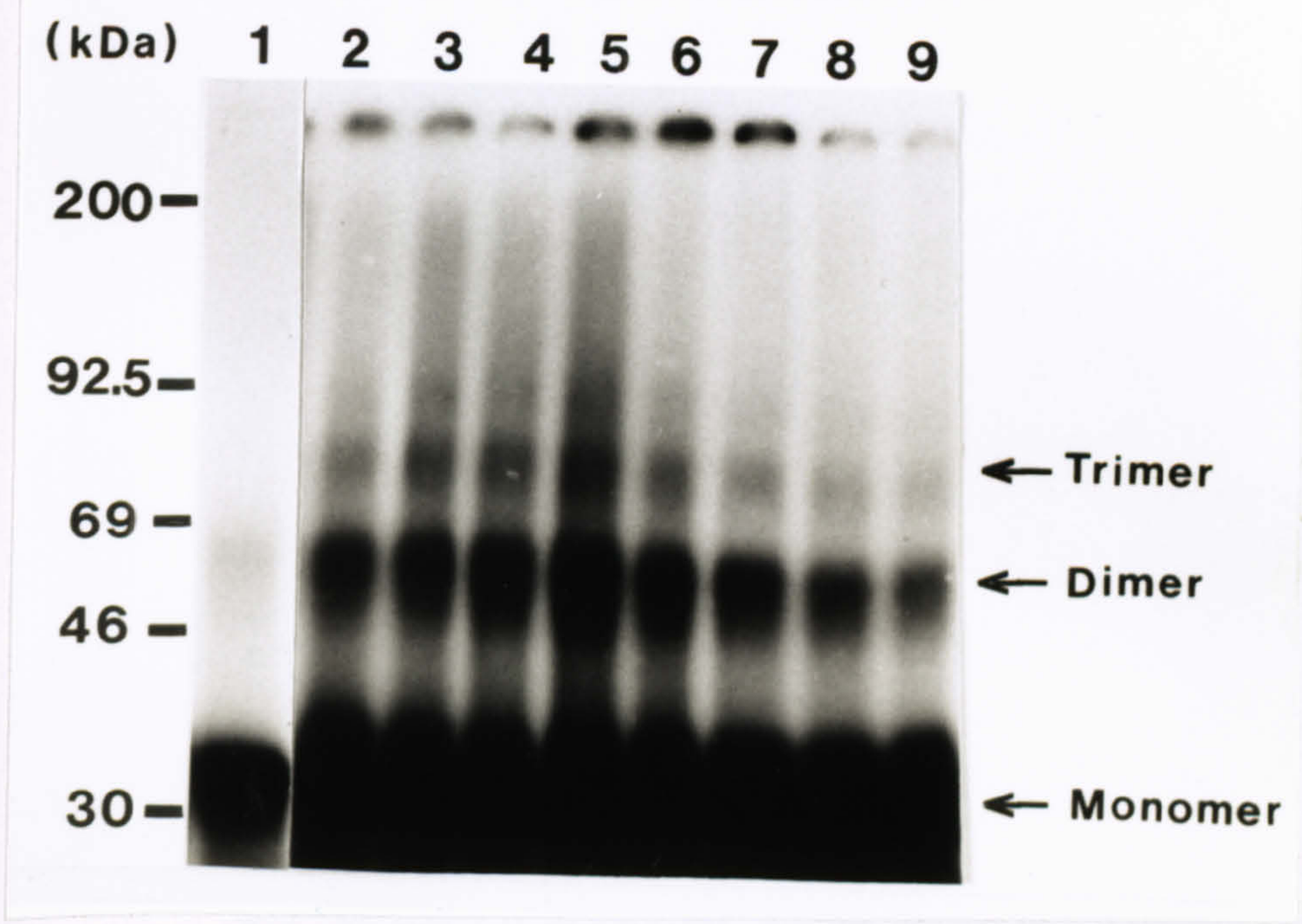


Fig. 3-14 Protein concentration effect on crosslinking of CD23.
The concentration of [^{125}I]-labelled CD23 in the reaction mixture was kept constant (2 µg/ml) while the concentration of the added unlabelled protein was increased by increments of 2 µg/ml; lane 1, no crosslinker added, lane 2, no unlabelled CD23 added, lane 3-9, incremental increase of unlabelled protein added: 2, 4, 6, 8, 10, 12 and 14 µg/ml, respectively. The concentration of EDC was kept constant at 100 mM; the positions of different molecular forms of CD23 and the standard molecular weight protein markers in the autoradiograph are marked.

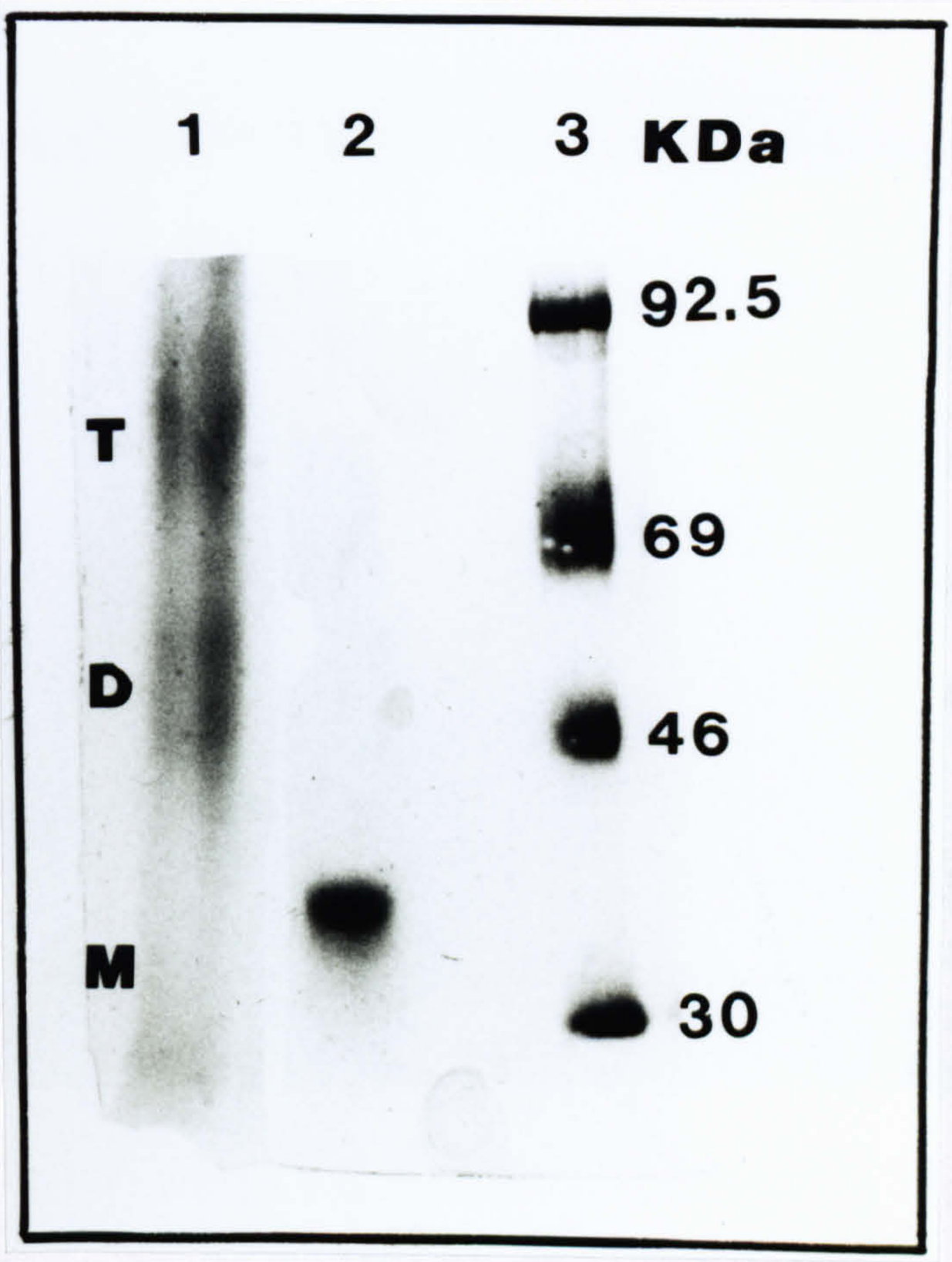


Fig.3-15 Crosslinking of CD23 at the protein concentration of 50 µg/ml.
Above is a picture of a silver stained gel of CD23 crosslinked with EDC at the protein concentration of 50 µg/ml (lane 1) and the negative control of crosslinking (no EDC added, lane 2). The experimental conditions were the same as in the previous experiments except that the optimal concentration of EDC for this concentration of the protein was 50 mM. The positions of CD23 trimer (T), dimer (D) and monomer (M) are marked.

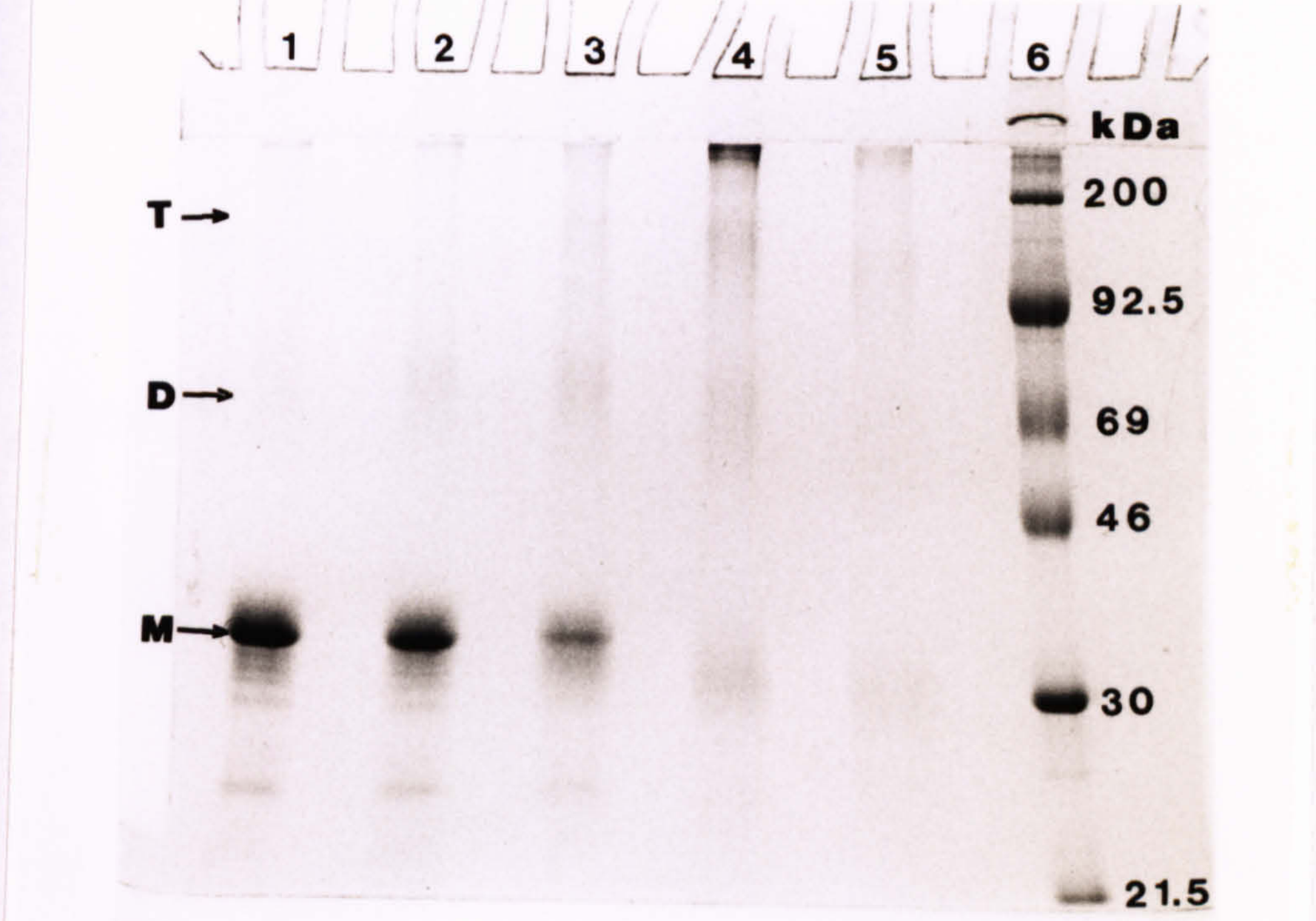


Fig.3-16 Crosslinking of CD23 at high protein concentration (1 mg/ml).
The Coomassie stained gel shown above is an attempt to demonstrate the concentration dependent oligomerisation of CD23 by chemical crosslinking at high concentration of the protein; unfortunately, most of the protein was converted into non-specific aggregates. The concentration of EDC in the experiment had been increased by 5 mM increment, from 0 (lane 1) to 20 mM (lane 5); the positions of CD23 trimer (T), dimer (D) and monomer (M) are marked.

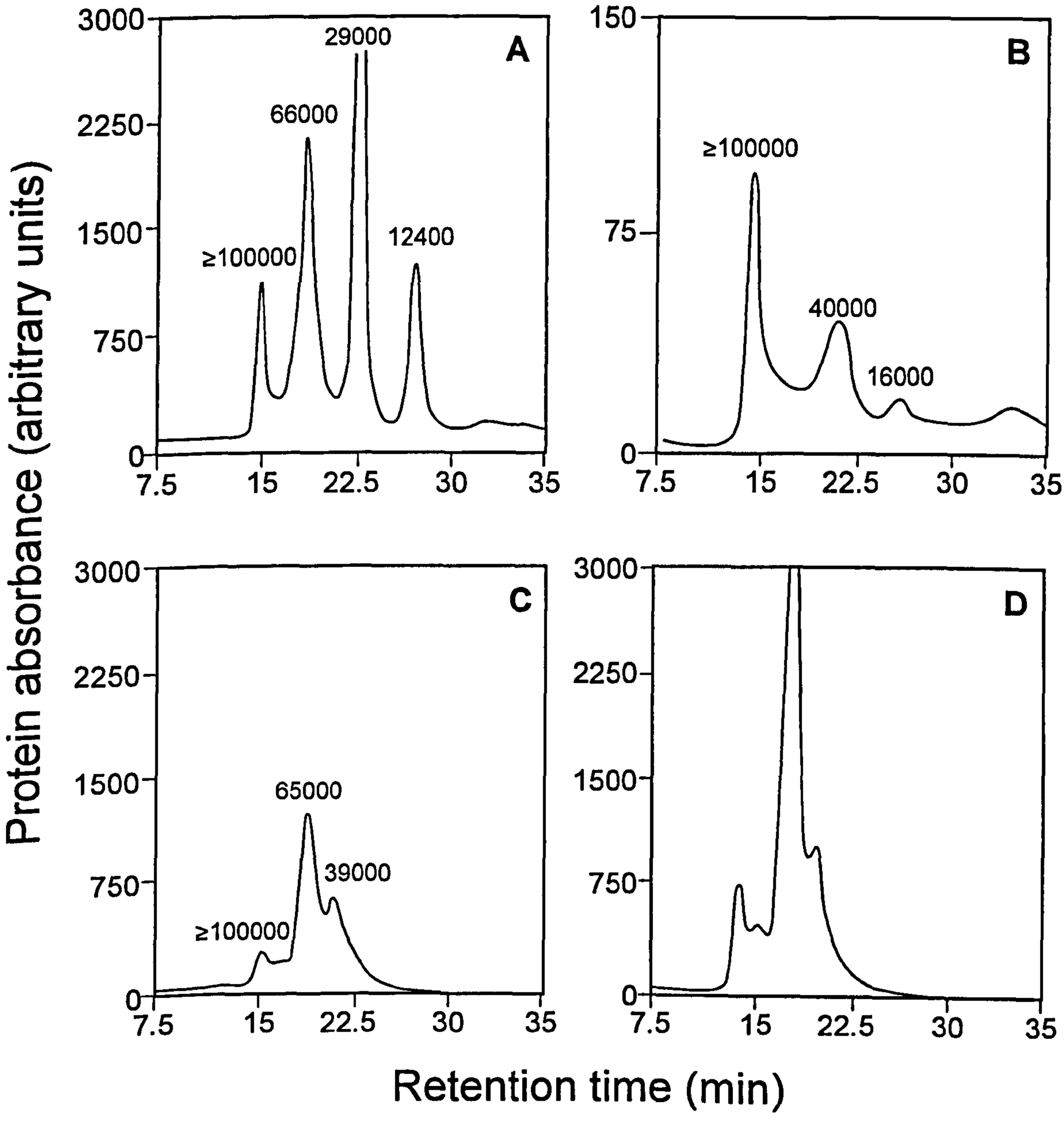


Fig.3-17 HPLC analysis of CD23 on Superdex S-75 column (Pharmacia).
A) The column was calibrated with standard molecular weight proteins (alcohol dehydrogenase, Mr 150000; BSA, Mr 66000; carbonic anhydrase, Mr 29000; cytochrome c, Mr 12400); the first marker (alcohol dehydrogenase) was used to indicate the exclusion limit of the column which is about 100000 (effective separation range is 3000-75000); B) native full length CD23 isolated from RPMI 8866 cells, at the concentration 0.033 mg/ml; C,D) recombinant CD23 at the concentration 0.33 and 1.33 mg/ml, respectively; the figures above the protein peaks indicate the calculated molecular weights according to the standard curve obtained from the profile shown in section *A* of the figure.

volume of the column and therefore is of the minimum molecular weight of 75000 but it is impossible to say whether this represents dimer or trimer of the protein. The second, smaller peak, however, has got the molecular weight of 40000 which corresponds to the size of native protein monomer.

On the basis of these HPLC profiles it is possible to measure the Stokes radius of CD23. Fig.3-18 shows the calibration curve for measurement of the Stokes radius of CD23 using Superdex 75 column, which was found to be 3.6 nm for the larger and 2.6 nm for the smaller protein fraction.

3.6.4. Calculation of the diffusion coefficient for CD23

The diffusion coefficient for the two protein forms observed on Superdex 75 profile were calculated from the equation:

$$D = KT / 6\pi\eta Rs \quad (1)$$

where K is Boltzman constant (1.380622 x 10^{-23} kg/m^2s^2), T is temperature (293 K), η is viscosity of the buffer used for gel filtration; this was practically same as the viscosity of water at room temperature, 1.00 g/ms), and Rs is the Stokes radius of the protein. The calculated values for D were 8.2 x 10^{-7} cm^2/s (smaller protein form) and 6.0 x 10^{-7} cm^2/s (larger form).

3.6.5. Measurement of the sedimentation coefficient of recombinant CD23

The sedimentation coefficient of CD23 was measured by centrifugation in the gradient of sucrose 5-20 % using the Beckman rotor SW 41. The protein sample was applied on top of the gradient (12 ml) and the centrifugation performed at 4 °C, at the speed of the rotor of 35000 rpm. The centrifugation time was 24-48 hours. After fractionation of gradients each fraction was analyzed for protein content and the typical profile obtained for CD23 is shown on Fig.3-19. Parallel to the CD23 sample, internal protein standards were run under exactly the same conditions and the profiles obtained were used for construction of

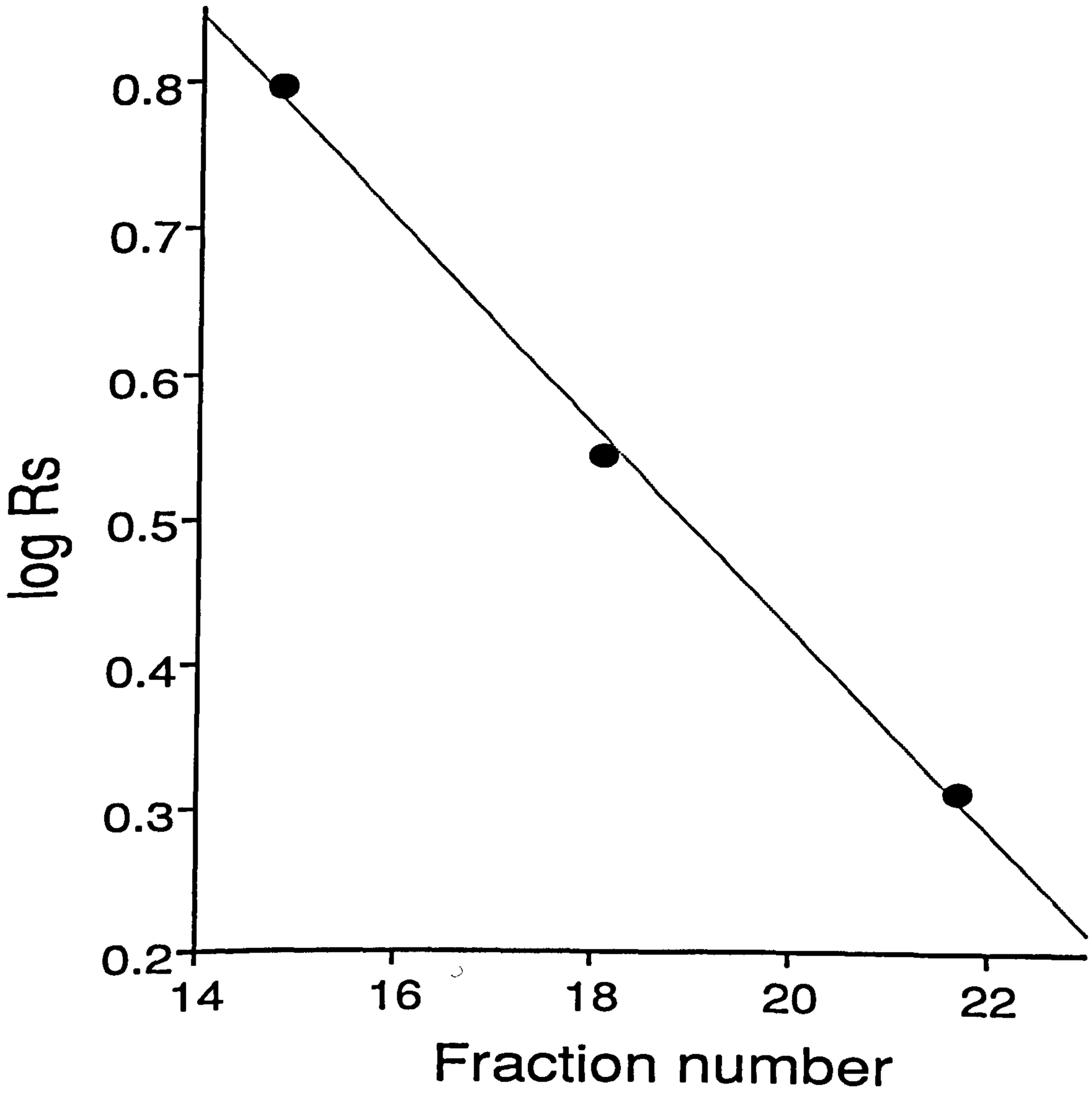


Fig.3-18 Measurement of the Stokes radius (Rs) for CD23.
The standard curve for measurement of Stokes radius of CD23 was constructed from the profile shown in Fig.3-17A; the values obtained were 3.6 nm (for the protein form of the apparent molecular weight of 65000 and 2.6 nm (for the protein form of the apparent molecular weight of 39000).

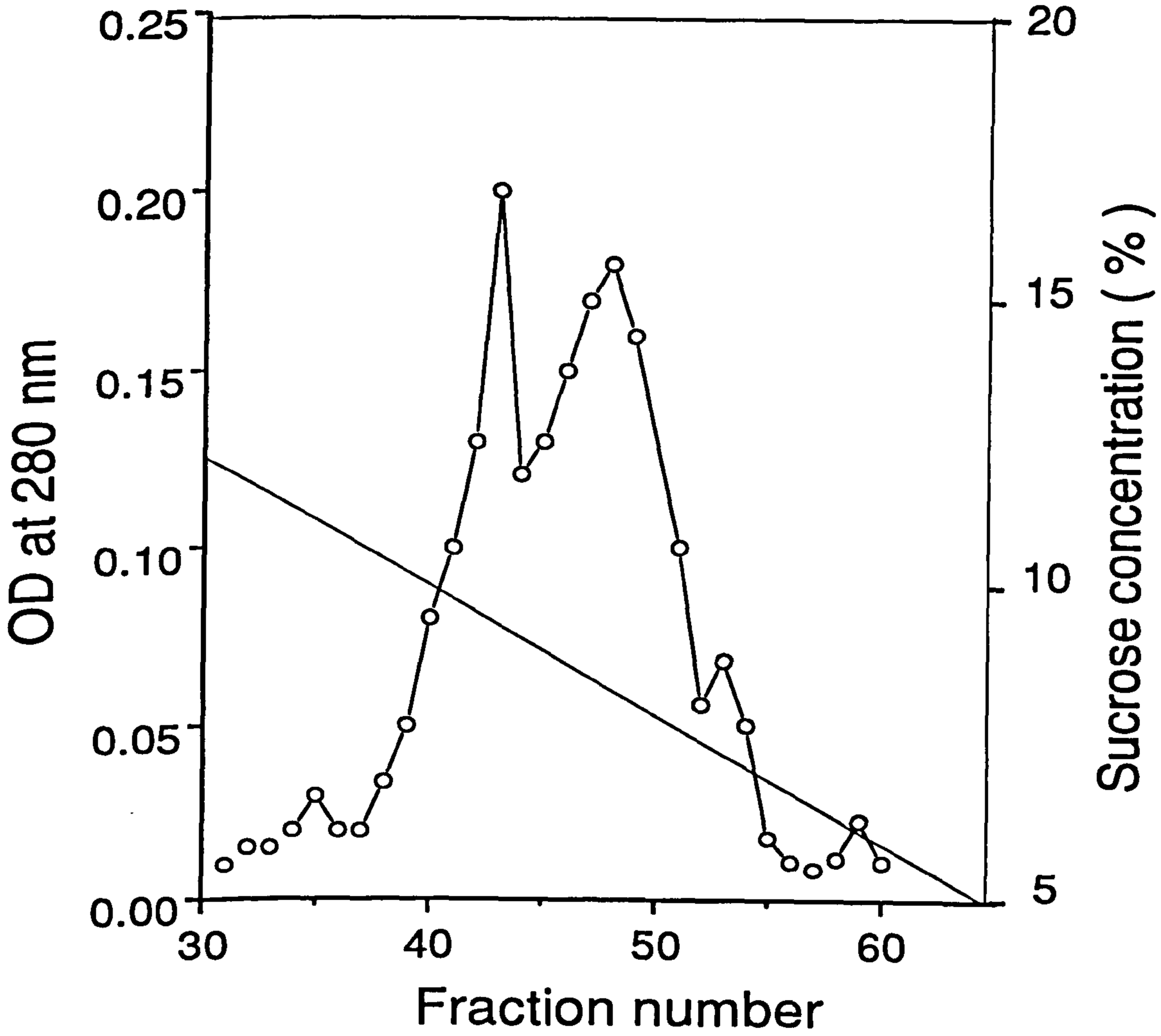


Fig.3-19 Centrifugation of recombinant CD23 in the gradient of sucrose concentration 5-20 %. CD23 sample (1 mg/ml, 0.1 ml) was applied on top of the sucrose gradient (5-20 %, 12 ml total volume) and the centrifugation performed at 35000 rpm in a Beckman rotor type SW 41, at 4 °C. Following 36 h centrifugation, the gradient was fractionated into 60 samples (0.2 ml) and the fractions were analyzed by spectrophotometry. Two major protein peaks were detected; the gradient of sucrose concentration is indicated by the diagonal line.

the standard curves used for determination of sedimentation coefficient ($S_{20,w}$) of CD23. A representative of these curves from 5 independent experiments is shown on Fig.3-20. The corresponding mean values for $S_{20,w}$ of the two protein forms of CD23 observed on the profile in Fig.3-19 were 3.5; SD: 0.17321; N = 5 (for the larger protein form) and 2.0 ; SD: 0.09231; N = 5 (for the smaller protein form). Neither of the two protein forms contains degradation products as confirmed by SDS-PAGE (not shown).

3.6.6. Calculation of the molecular weight of recombinant CD23

On the basis of measured diffusion and sedimentation coefficients for CD23 the molecular weight was calculated from the Svedberg's equation:

$$Mr = RTS / D(1-\bar{\upsilon}\rho) \quad (2)$$

where R is gas constant (8.314 J/K mol), S is sedimentation coefficient, $\bar{\upsilon}$ is partial specific volume of CD23 (0.7196 g/ml) and ρ is the density of CD23 containing fraction from the sucrose gradient (1.0299 g/ml). The calculated mean values for Mr were 55000; SD: 3000; N = 5) for the larger protein form and 32000; SD: 2400; N = 5 (for the smaller protein form).

3.6.7. Dynamic light scattering analysis of recombinant CD23

The experiment was performed in a DynaPro-801 instrument (Protein Solutions, High Wycombe, UK). CD23 solution at the protein concentration of 2 mg/ml was prepared, injected into the sample cell and illuminated by laser. The obtained data include several parameters such as radius of the protein (nm), polydispersity of the solution (nm), estimated molecular weight, baseline (corresponds to the quality of the obtained fit for the applied model) and sum of squares, SOS (refinement of the fit). From these parameters it is possible to distinguish between single protein form and mixture of two or more forms, and in the case where the polydispersity is negligible it is possible to measure the molecular weight of the protein. In the case of CD23 the following data were obtained:

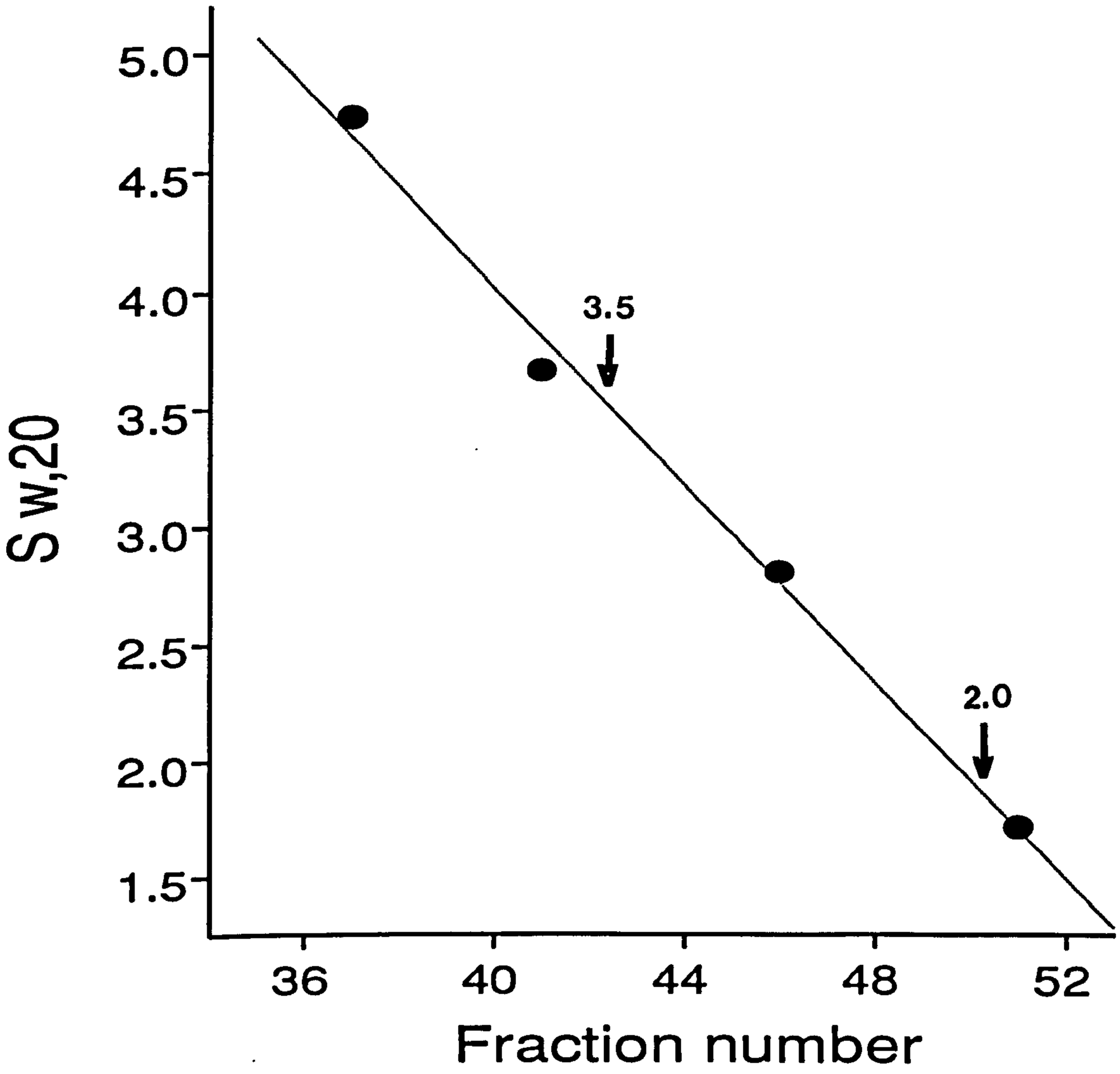


Fig.3-20 Standard curve for measurement of sedimentation coefficient ($S_{20,w}$) of CD23. The standard curve for determination of $S_{20,w}$ of CD23 was constructed from the sedimentation profiles obtained for proteins of known molecular weights and $S_{20,w}$ coefficients: BSA (Mr 66000, $S_{20,w}$ 4.73), ovalbumin (Mr 45000, $S_{20,w}$ 3.66), carbonic anhydrase (Mr 29000, $S_{20,w}$ 2.8), cytochrome c (Mr 12400, $S_{20,w}$ 1.71). The $S_{20,w}$ values for the two CD23 forms shown in Fig.3-19 were 3.5 (larger) and 2.0 (smaller).

Table 3-2: Dynamic light scattering analysis of recombinant CD23.

Reading number	Radius (nm)	Polydisper-sity	Estimated Mw	Baseline	Sum of squares
1	4.8	negligible	136000	1.003	4.672
2	4.7	negligible	129000	1.005	4.192
3*	5.7	3.1	200000	1.018	8.119
4	4.9	negligible	138000	0.999	2.988
5	4.3	negligible	101000	1.005	7.613

* This reading was exempted from the calculations.

As can be seen in Table 3-2 one of the readings gave unacceptable polydispersity factor and was excluded from the calculation of the average molecular weight which was found to be 126000. This is close to the theoretical molecular weight of CD23 tetramer (120000). The Baseline parameter is interpreted as follows: 0.997-1.001, distribution is monomodal; 1.002-1.005, distribution is primarily monomodal but there may be a small amount of larger size molecules; >1.005, size distribution is polydisperse. Thus, although CD23 at the concentration of 2 mg/ml behaves as a primarily monodisperse system the parameters for baseline are close to the transition range for polydisperse system. The SOS parameter can be interpreted in similar manner. These data are discussed in detail in Chapter 6.

3.7. Summary and conclusions

In this chapter, the expression of human soluble CD23 in *E. coli* is described. The extracellular sequence of CD23 was subcloned into a bacterial expression vector and large quantities of the recombinant protein were obtained (50 mg/l of cell culture). An optimal protocol for renaturation of the bacterial product was designed, based on the method of

mixed disulphide formation between the cysteine residues of protein and oxidised glutathione. CD23 possesses 8 cysteine residues capable of forming 4 disulphide bridges, all of which are located in the lectin domain of the protein. This is a certain difficulty in any attempt to refold the denatured protein, as the probability of incorrect cysteine pairing is high in addition to the nonspecific intermolecular pairing. Several different protocols were applied with variable success. In most cases the amount of the aggregated material at the end of refolding procedure was unacceptably high as compared to the amount of soluble protein (> 80 %). However, the protocol described in this work has been proved to work reasonably well with at least 50 % recovery of the soluble protein.

It has been further shown that the refolded protein binds to IgE (Ka = 2.1 x 10^6 M) and moreover, it can greatly enhance the internalization of IgE-CD23 complexes on B cells presumably through a noncovalent association with membrane CD23 (S. Karagianis, Ph.D thesis). In the following chapter the evidence will be presented showing that it also binds to the second ligand, CR2. Therefore, the *E. coli* expressed CD23 displays the two most important activities and could be used for relevant biophysical studies carried out in this work. It has been demonstrated by HPLC and sucrose gradient centrifugation method that the recombinant CD23 preparation consists of at least two distinct molecular forms of the corresponding molecular weights of 55000 and 32000, respectively, as calculated from the Svedberg's equation. These two forms correspond well with the expected molecular weights of the protein dimer (60000) and monomer (30000). In addition to these two protein forms, a larger protein form with the minimal calculated molecular weight of 70000 was detected on an HPLC column and this may represent protein trimer of CD23. However, no trimer or any other form apart from dimer and monomer were detected by sucrose gradient centrifugation.

The previously published results of Beavil *et al.*, 1995, showed that soluble CD23 is capable of forming protein trimers which can be detected by chemical crosslinking. In this work, it has been shown that the recombinant CD23 from *E. coli* does indeed form protein trimers, according to the results obtained by crosslinking of the protein. Nevertheless, it was not possible to accurately demonstrate the presence of this protein form by any of the size exclusion limit methods applied in this work. Instead, the gathered

experimental evidence indicates the possibility of an equilibrium between the three forms of the protein (monomer/dimer/trimer) and a possible higher level association of CD23; this is discussed in more details in Chapter 6 (sections 6.3-6.5).

CHAPTER 4: INTERACTION OF CD23 AND CR2

4.1. Introduction

The interaction of CD23 and CR2 was first described by Aubry *et al.*, 1992 by using the technique of fluorescent CD23-liposomes which were able to bind to some but not all CR2 positive cells. The same group of researchers and others then proposed several functional implications for this interaction described in Chapter 1, section 1.3.3. The published literature on this subject reveals that no other form of CD23, natural or recombinant, apart from the native intact protein was directly shown to interact with CR2. As for the nature of interaction between CD23 and CR2, it has been proposed that the carbohydrates on CR2 may be involved, although the peptide structure of SCRs 1 and 2 contribute to the binding as well (Aubry *et al.*, 1994). One of the aims of this study was, therefore, to test the ability of recombinant soluble CD23 to bind to both native and recombinant CR2 and to measure the affinity constant for the interaction.

The experimental approach to resolve this question was to isolate and purify the native forms as well as to generate and characterize the recombinant soluble forms of both proteins and to compare the affinity constants for the interaction between different forms of CD23 and CR2. In Chapter 3, the production and characterization of recombinant soluble CD23 from *E. coli* has been described. This Chapter describes the production of recombinant soluble CR2 in insect cells using the baculovirus expression system. The recombinant CR2 baculovirus expression vector was readily available and was kindly provided by G. Nemerow (The Scripps Research Institute, La Jolla, CA). The expressed protein was previously reported to be capable of binding C3dg and inhibiting Epstein-Barr virus infection (Nemerow *et al.*, 1990), and the protein appeared to be glycosylated according to the increase in the molecular weight as compared with the expected molecular weight of non-glycosylated polypeptide precursor. The glycosylation of recombinant CR2 was an important consideration for studying the interaction with CD23 since it was reported that the carbohydrate structures on CR2, as well as the peptide backbone of SCRs 1 and 2, mediate the binding (Aubry *et al*, 1992; Aubry *et al.*, 1994).

It is, however, as yet unclear why CR2 from only some types of cells, or just one particular isoform of the protein from the same type of cell bind,to CD23 (Aubry *et al*, 1992). If the contribution of the peptide structures of the SCRs 1 and 2 to the total binding may be as high as 50 % (Aubry *et al.*, 1994), one might expect that all CR2 populations would be able to bind CD23 with at least 50 % capacity, as compared to the isoforms with the most appropriate carbohydrate structures. Therefore, an attempt has been made to find out whether CR2 expressed in insect cells, which significantly differ in their glycosylation potential from the animal cells, is capable of binding CD23. In this Chapter, the presented results suggest that there is a detectable interaction between the recombinant soluble CR2 and CD23. In addition to that, this Chapter presents the evidence of simultaneous binding of CR2 and IgE to the dimeric CD23.

4.2. Binding of native and recombinant CD23 to CR2 on cell surface

4.2.1. Isolation and purification of intact, 45-kDa form of CD23

The membrane-associated form of CD23 (45-kDa) was isolated from RPMI 8866 cells by detergent extraction in the presence of a cocktail of protease inhibitors as described by Cairns and Gordon, 1990. Initially, the cells were prepared on a small scale in cell culture flasks but for the larger quantities of CD23 the cells were grown in a 20 l cell culture fermenter (Pfizer). In this way 1 x 10^{11} cells were prepared and used for isolation of CD23. Two detergents were tested for lysing the cells: Nonidet NP-40 and octyl-glucopyranoside (OGP). Although Nonidet-NP-40 seems to produce higher yield of the extracted protein it was found that this detergent is not appropriate because of the difficulty of removing it prior to the liposome preparation. The OGP-extracts of cells were run over anti-CD23 antibody columns (MHM6 or mAb-25) and the bound material eluted with 0.2 M glycine, pH 2.3, 0.2 % OGP, 0.2 mM TLCK. CD23 prepared in this way is mostly intact (Fig.4-1) but in some cases detectable degradation still occured (not shown).

4.2.2. Preparation of fluorescent CD23-liposomes

The fluorescent CD23-liposomes were prepared essentially as described by Pochon *et al.*,

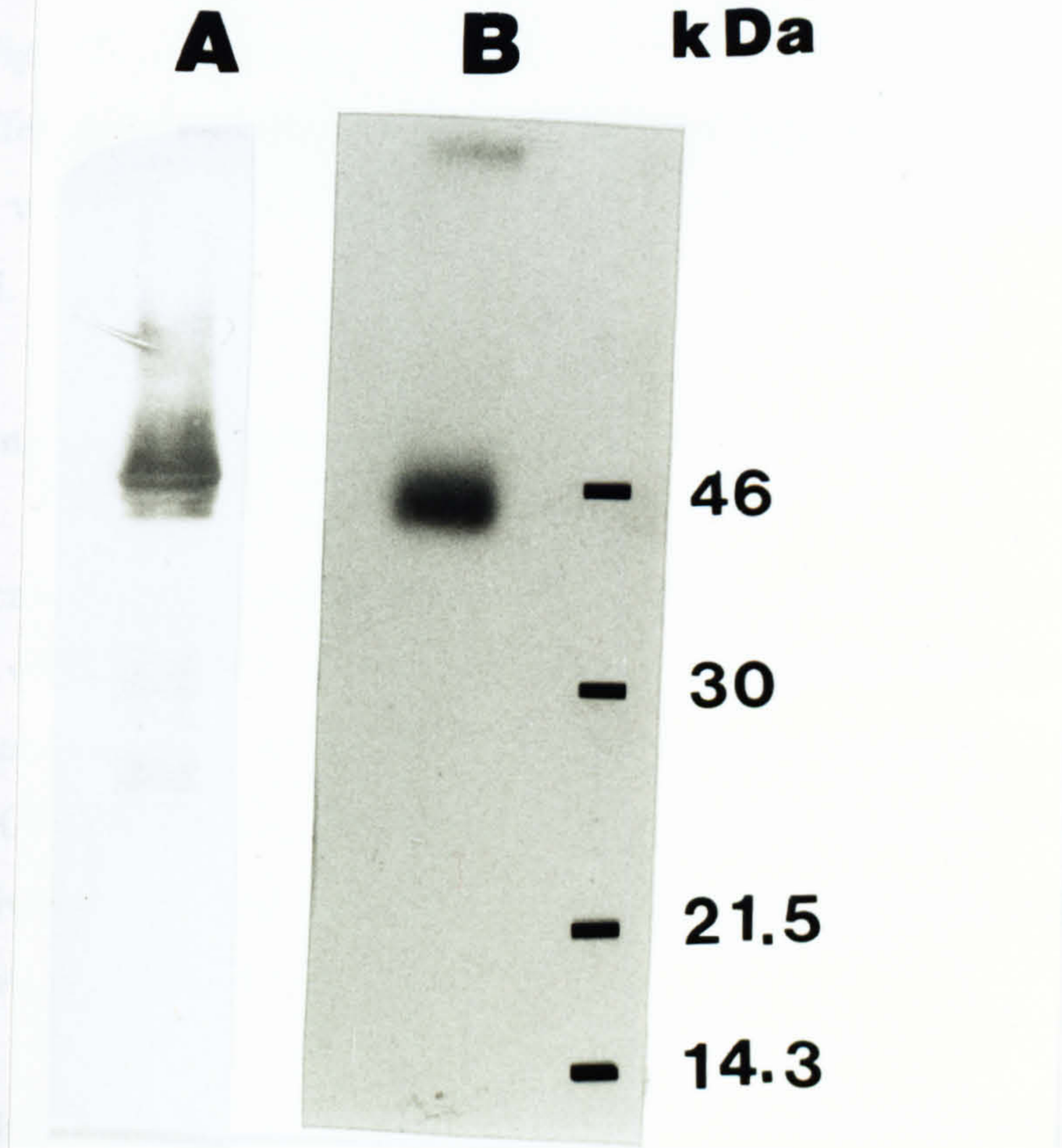


Fig.4-1 Isolation and purification of intact 45 kDa CD23 from RPMI 8866 cells. The native intact CD23 was purified from the lysates of RPMI 8866 cells by affinity chromatography on MHM6 column, in the presence of 0.2 mM TLCK. The isolated material was probed with anti-CD23 antibody BU-38 by Western blotting (a) and the purity was verified by autoradiography of an iodinated sample of CD23 (b). No detectable degradation of the protein could be observed in either case.

1992 with a few modifications described in Chapter 2. To ensure that no free CD23 or fluorescent label has remained in the preparation the liposomes were pelleted by centrifugation at 40000 x g for 2 h, at 4 °C. Some of the liposome pellet was used for electron microscopy analysis to show the actual incorporation of the protein into the lipid structure (Fig.4-2) and the rest of the preparation was resuspended in the HBS-Ca^{2+} binding buffer and used for FACs analysis. The negative control in all subsequent experiments with CD23-liposomes was a fluorescent liposome preparation with no CD23 incorporated.

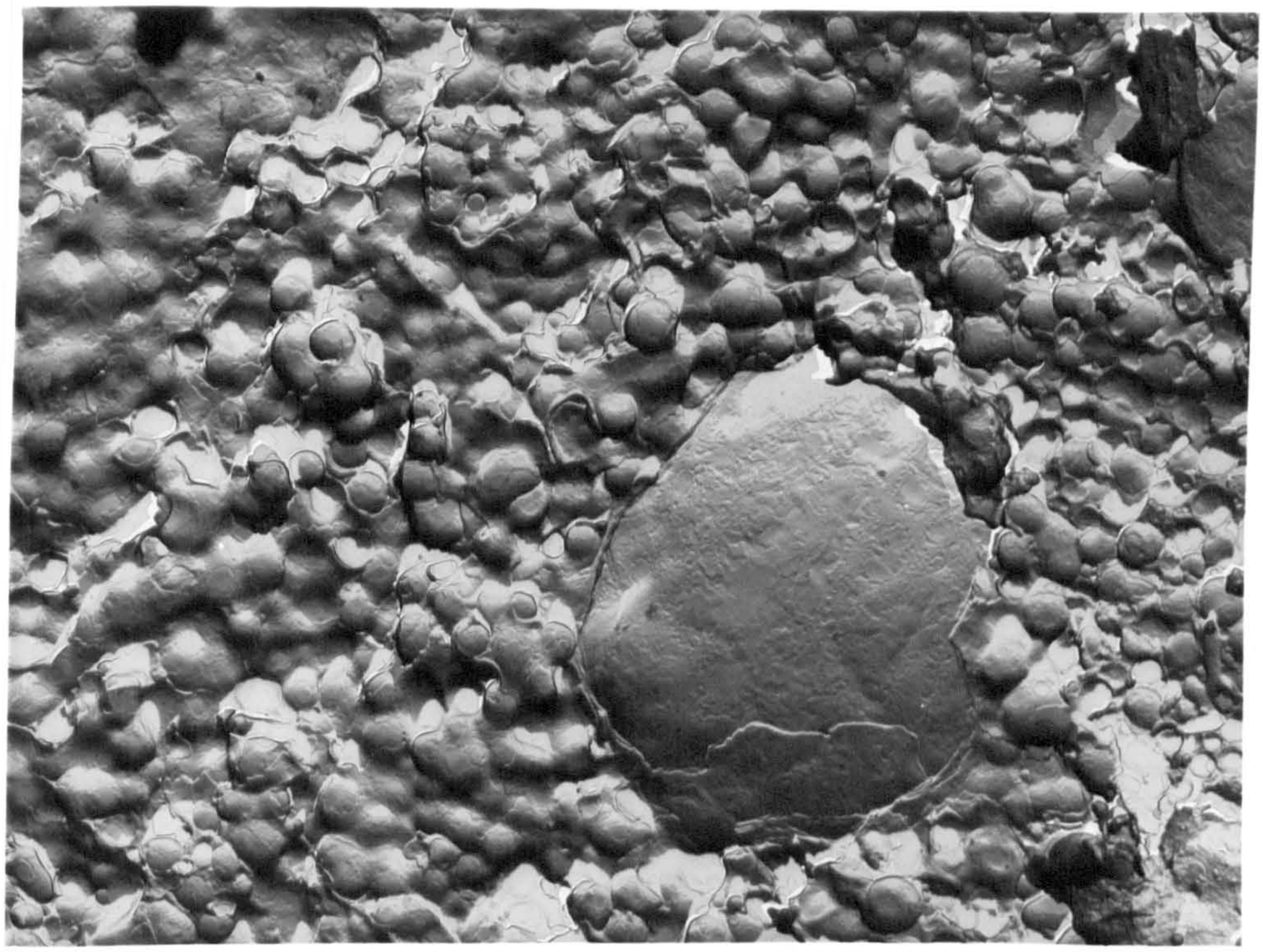
4.2.3. Binding of CD23-liposomes to CR2 on RPMI 8226 cells

The fluorescent CD23-liposomes were able to bind to RPMI 8226 cells which are CR2 positive and were previously shown to be the most appropriate cell type tested for binding of CD23-liposomes (Pochon *et al.*, 1992). After 2 h incubation of 1 x 10^6 cells in the 5-fold diluted CD23-liposomes preparation most of the cells stained positively as compared to the negative control. The FACs profile of binding of CD23-liposomes to RPMI 8226 cells is shown in Figure 4-3A.

4.2.4. Inhibition of CD23-liposomes binding to RPMI 8226 cells by native CR2

A small amount of native CR2 was isolated from 1 x 10^{10} RPMI 8226 cells and purified by affinity chromatography on anti-CR2 antibody HB5 column and used as the competitor in the CD23-liposomes binding assay. As a direct demonstration that CR2 is indeed the new ligand for CD23, the capacity of native CR2 prepared in this way to inhibit the binding of CD23-liposomes to RPMI 8226 cells is shown in Fig.4-3B. The reason that this inhibition is not complete is probably the low concentration of CR2 in the preparation (higher concentration of protein could not be used because of extensive cell lysis caused by the increase in concentration of the detergent present in the preparation).

A



B

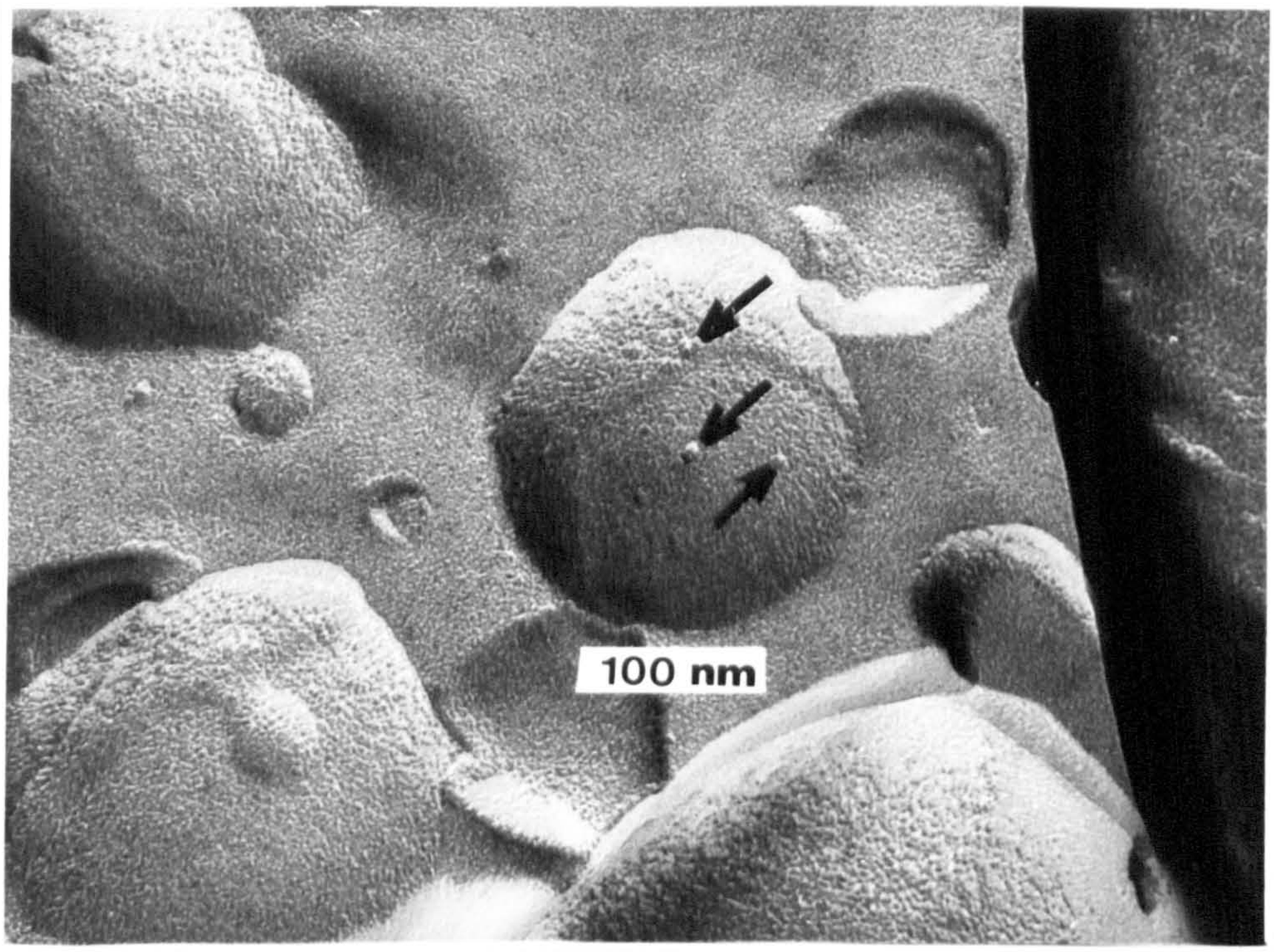


Fig.4-2 CD23-liposomes.
CD23 liposomes were pelleted, frozen and the horizontal sections examined by electron microscope; under the magnification 1:9800 the liposomes appear as tightly packed spherical structures rather homogenous in size (A); under the magnification 1:130000 it is possible to detect the protein spots on the lipid surface of liposomes (B); on the shown photograph the white bar represent 100 nm and the liposome just above the bar is approximately 200 nm in size; CD23 is represented by the small light spots that can be seen on the surface of this liposome.

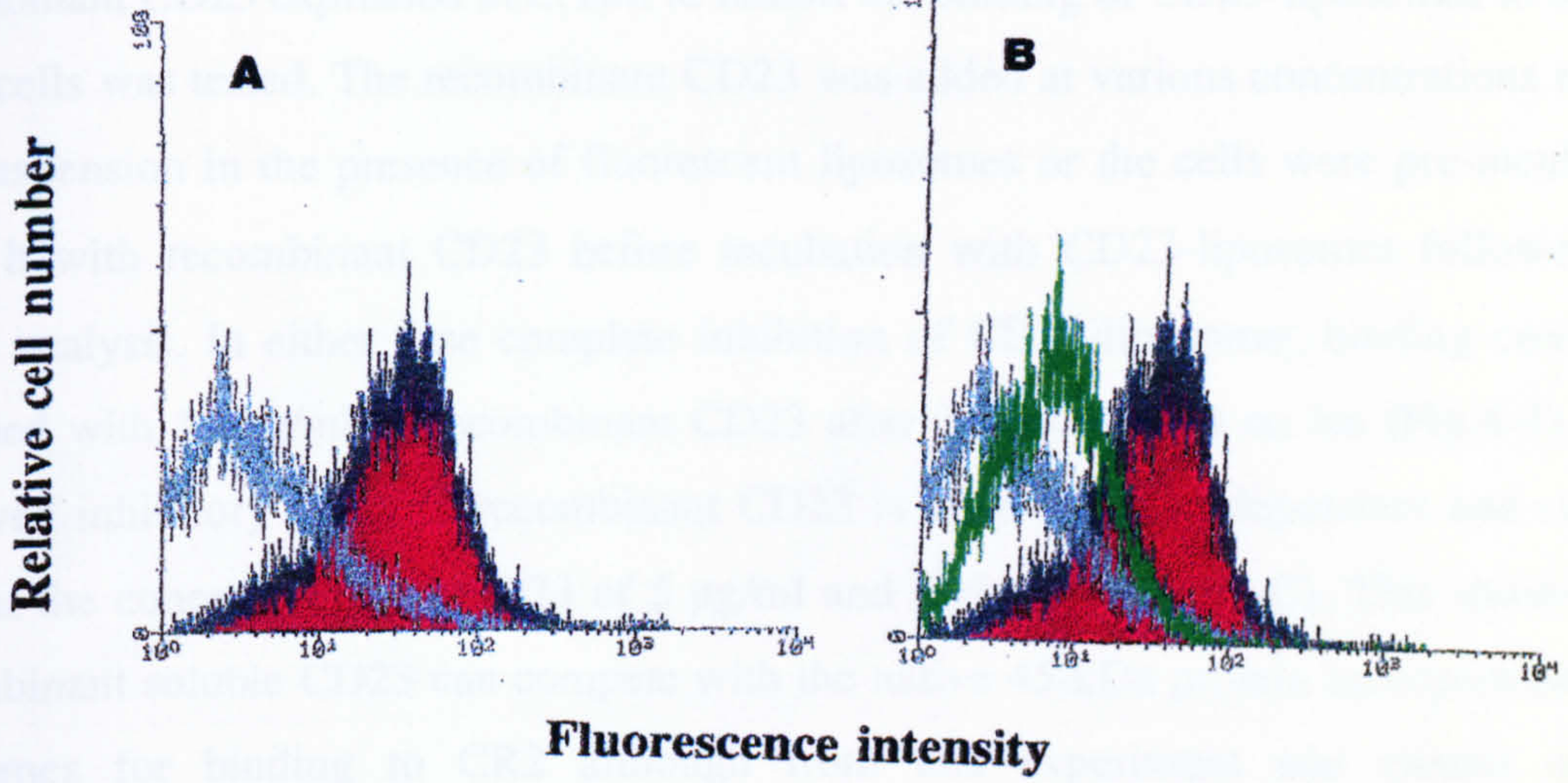


Fig.4-3 CD23 liposomes bind to RPMI 8226 cells and the binding can be inhibited by native CR2 isolated from the same cells.
Five-fold diluted fluorescent CD23-liposomes were added to the suspension of RPMI 8226 cells (1 x 10^6) and the incubation carried out on ice for 2 h; after that the cells were washed twice with the binding buffer (HBS, 2 mM Ca^{2+}, 0.1 % Na-azide) and examined for fluorescence by FACs (a); the open peak represents binding of negative liposomes that were prepared in the exactly same way as the CD23-liposomes except that no CD23 was added, and the red peak represents the binding of CD23-liposomes to RPMI 8226 cells. In a parallel assay the detergent solubilised native CR2 isolated from the same cells by affinity chromatography on HB5 column, was diluted to reduce the concentration of the detergent to below 0.1 % and added together with CD23-liposomes to the suspension of cells; the green peak represents the binding of CD23-liposomes to RPMI 8226 cells in the presence of purified CR2. In this and all following FACs assays 10000 cells were counted.

4.2.5. Inhibition of CD23-liposome binding to RPMI 8226 cells by recombinant CD23

In order to find out whether CD23 fragments can bind to CR2, the capacity of recombinant CD23 expressed in *E. coli* to inhibit the binding of CD23-liposomes to RPMI 8226 cells was tested. The recombinant CD23 was added at various concentrations to the cell suspension in the presence of fluorescent liposomes or the cells were pre-incubated for 2 h with recombinant CD23 before incubation with CD23-liposomes followed by FACs analysis. In either case complete inhibition of CD23-liposome binding could be obtained with 10 µg/ml of recombinant CD23 after 2 h incubation on ice (Fig.4-4). The observed inhibitory effect of recombinant CD23 is concentration dependent and can be seen at the concentration of CD23 of 5 µg/ml and higher (Fig.4-5A-C). This shows that recombinant soluble CD23 can compete with the native 45-kDa protein incorporated into liposomes for binding to CR2 although from this experiment one cannot obtain quantitative measure of the relative affinities. The recombinant CD23 was not able to inhibit binding of CD23-liposomes to the cells once they were already incubated with CD23-liposomes (Fig.4-5D-F). It was found that 30 min incubation of the cells with CD23-liposomes was sufficient to eliminate almost completely the inhibitory effect of recombinant CD23 while longer incubation (1.5 h) completely diminished this effect. Although the recombinant CD23 is able to compete with the native protein for binding to CR2 the affinity seems to be lower than that of the native CD23. The final concentration of the native CD23 in the assay was 1 µg/ml or less (depending on the amount of the protein that was not incorporated into the liposomes and was subsequently removed by centrifugation of the liposomes) and the addition of the recombinant CD23 at the same concentration does not cause 50 % inhibition, as would be expected in the case of the same affinity. To achieve 50 % inhibition the required concentration of CD23 was 5 µg/ml while for the complete inhibition it was necessary to add 10 µg/ml of CD23.

4.2.6. Inhibition of CD23-liposome binding to RPMI 8226 cells by IgE

The evidence presented to date in the literature on the inhibition of the CD23-CR2

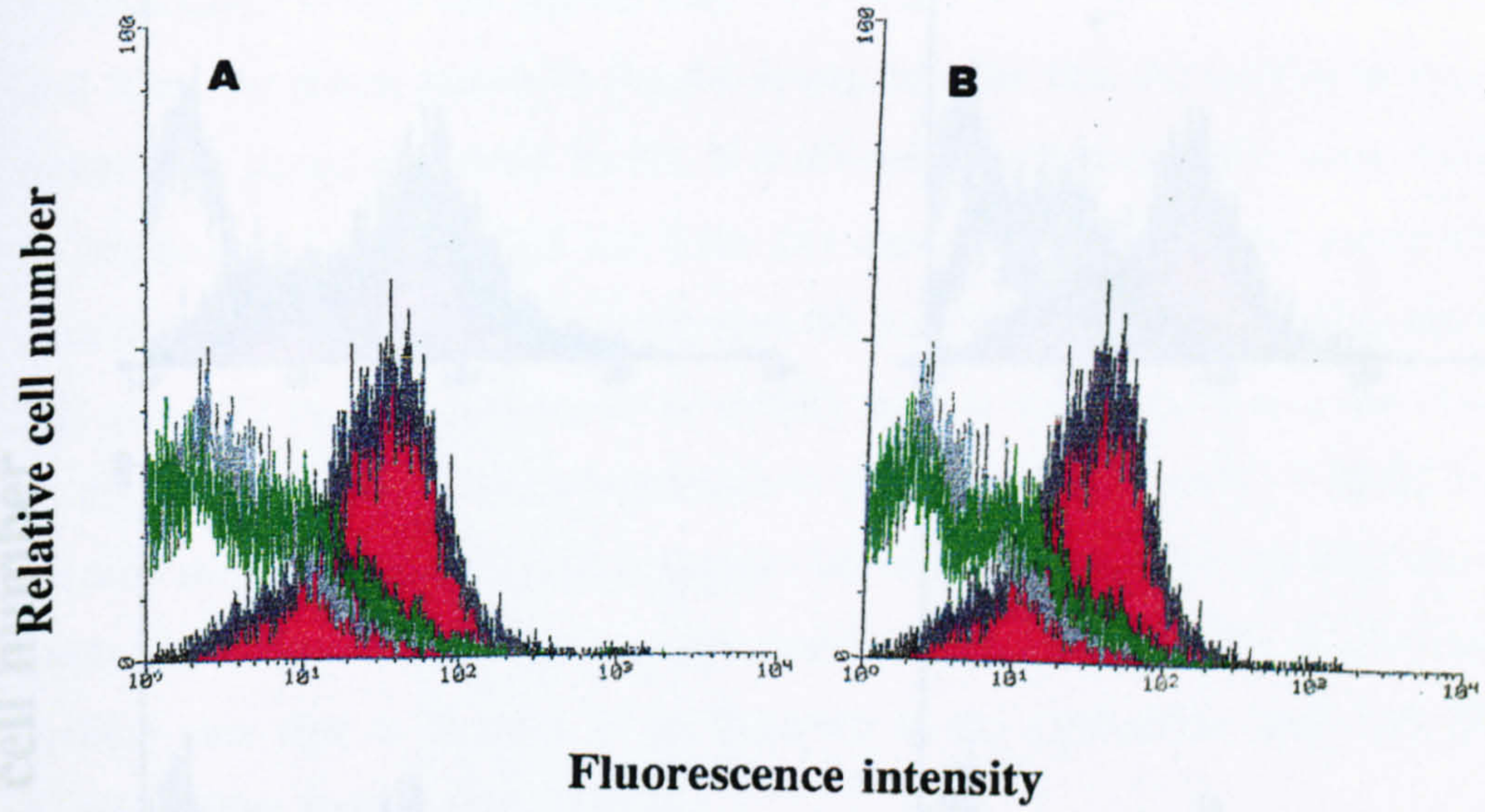
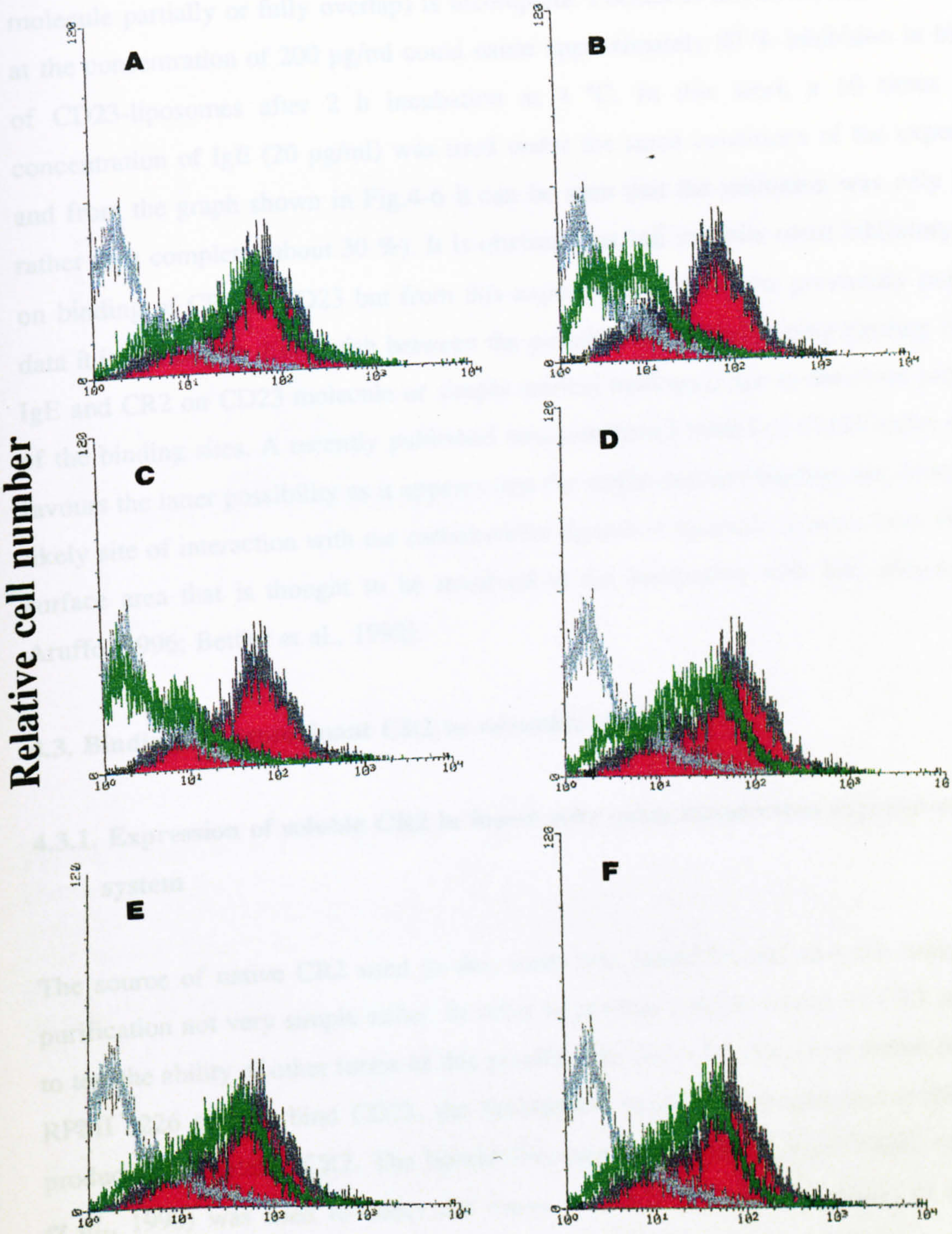


Fig.4-4 Recombinant CD23 inhibits the binding of CD23-liposomes to RPMI 8226 cells.

Recombinant CD23 at the concentration of 10 µg/ml was added to the suspension of RPMI 8226 cells 2 h before addition of CD23-liposomes (A) or together with the liposomes (B) and the cells were incubated for further 2 h; after washing off the unbound liposomes the cells were assayed by FACs; the open light-grey peak represents the binding of negative liposomes, the filled red peak binding of CD23-liposomes and the green peak the binding of CD23-liposomes in the presence of recombinant CD23. The fluorescence intensity in this and all other FACs experiments is given in logarithmic scale.

Fig.4-5 Inhibition of CD23-liposome binding to RPMI 8226 cells by recombinant CD23: concentration dependence and relative affinity of binding.
Different concentrations of recombinant CD23 were analyzed for the capacity to inhibit the binding of CD23-liposomes to RPMI 8226 cells (green peak); 1 µg/ml (approximately equal to the concentration of native CD23 in the reaction mixture) caused no significant inhibition (A), 5 µg/ml caused approximately 50 % inhibition (B) and 10 µg/ml completely inhibited binding of CD23-liposomes (C). Pre-incubation of the cells with CD23-liposomes for 30 min reduced the inhibitory effect of recombinant CD23 (10 µg/ml) by 70 % (D); further prolongation of the pre-incubation time to 1 and 1.5 h, respectively, almost completely eliminated inhibitory effect of recombinant CD23 (E,F).


A
B
C
D
E
F
Relative cell number
Fluorescence intensity

interaction by IgE (which would indicate that the binding sites for IgE and CR2 on CD23 molecule partially or fully overlap) is incomplete. Pochon *et al.*, 1992, showed that IgE at the concentration of 200 µg/ml could cause approximately 80 % inhibition in binding of CD23-liposomes after 2 h incubation at 4 °C. In this work a 10 times lower concentration of IgE (20 µg/ml) was used under the same conditions of the experiment and from the graph shown in Fig.4-6 it can be seen that the inhibition was only partial rather than complete (about 30 %). It is obvious that IgE exhibits some inhibitory effect on binding of CR2 to CD23 but from this experiment or from the previously published data it is difficult to distinguish between the possibilities of overlapping binding sites for IgE and CR2 on CD23 molecule or simply sterical hindrance due to the close proximity of the binding sites. A recently published structure based model of CD23 lectin domain favours the latter possibility as it appears that the single calcium binding site which is the likely site of interaction with the carbohydrate ligands is spatially distinct from the large surface area that is thought to be involved in the interaction with IgE (Bajorath and Aruffo, 1996; Bettler et al., 1992).

4.3. Binding of recombinant CR2 to recombinant CD23

4.3.1. Expression of soluble CR2 in insect cells using baculovirus expression system

The source of native CR2 used in this work was rather limited and the isolation and purification not very simple either. In order to provide a better source of CR2 as well as to test the ability of other forms of this protein apart from the intact membrane form from RPMI 8226 cells to bind CD23, the baculovirus expression system was employed for production of soluble CR2. The baculovirus expression vector pAc373-CR2 (Nemerow *et al.*, 1990) was used to infect the insect cells Sf9 and the expression of CR2 was monitored over the period of 7 days. It was found that maximum expression of CR2 is reached after 5 days following infection (Fig.4-7), at which point about 50 % cells are dead. Further incubation does not improve the yield of CR2 significantly while the contamination of supernatant with cellular contents increases.

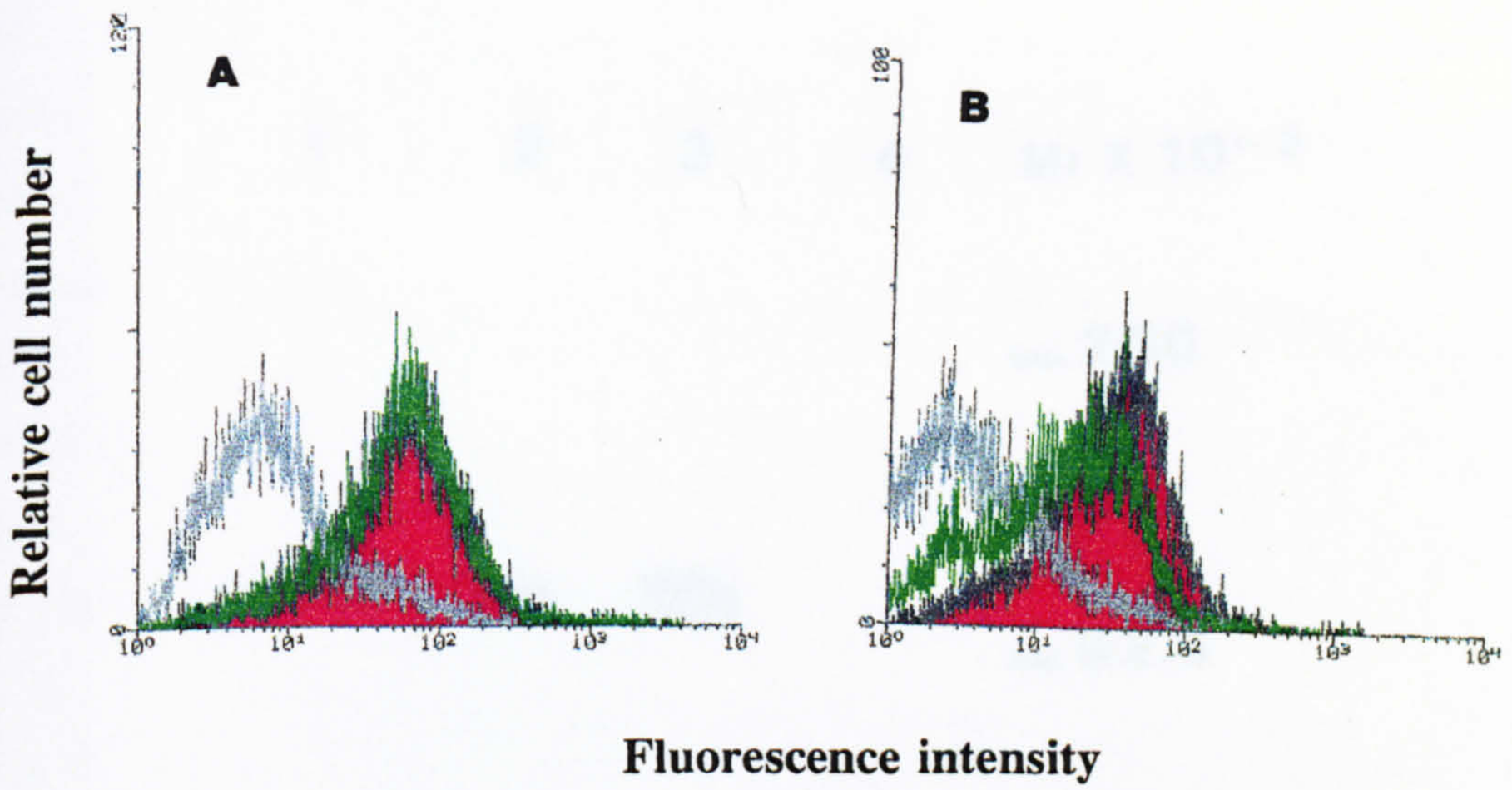


Fig.4-6 Inhibition of CD23-liposome binding to RPMI 8226 cells by IgE.
Human IgE at the concentration of 2 (A) and 20 (B) μg/ml was added to the suspension of RPMI 8226 and CD23-liposomes and the incubation on ice carried out for 2 h; the cells were washed twice with the binding buffer and examined for fluorescence; no detectable inhibition was observed with 2 μg/ml of IgE and a partial inhibition (20-30 %) was detected with 20 μg/ml of IgE (green peak).

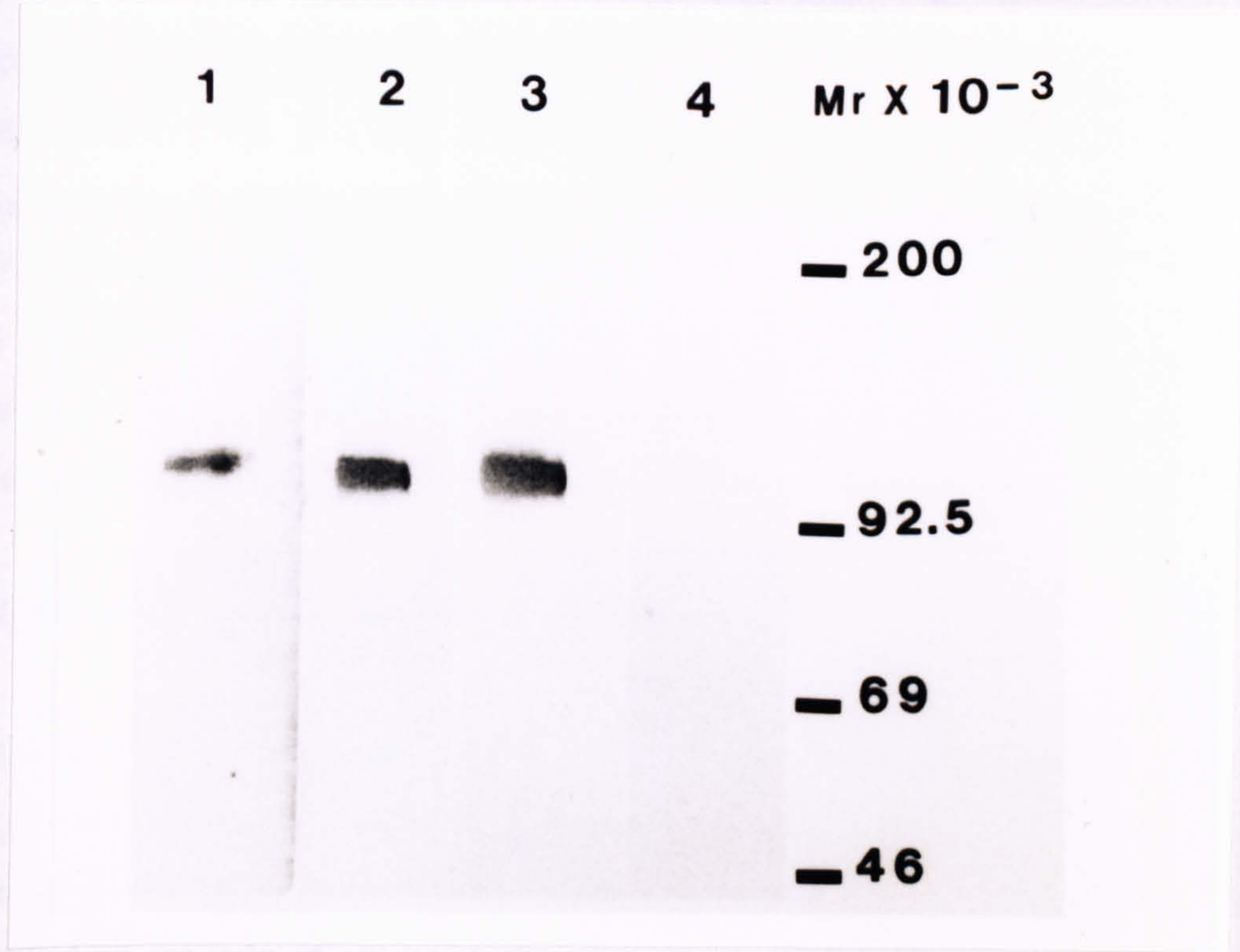


Fig.4-7 Expression of soluble CR2 in insect cells: time course for expression following the infection with recombinant CR2 baculovirus.
The above photograph is a Western blot of soluble CR2 containing insect cell supernatants (Sf9 cells) that were taken at different times following infection with the virus; lanes 1, 2 and 3 correspond to the time points 3, 4 and 5 days, respectively; after 5 days no further increase in the secreted CR2 was detected (not shown). Lane 4 is the negative control in the experiment (supernatant from non-infected cells); the antibody used for detection of CR2 was BU32.

4.3.2. Partial purification of recombinant CR2

Following 5 days incubation the cells were removed from the culture by centrifugation (3000 rpm, Sorvall GSA rotor) for 20 min and the supernatant was dialysed against 10 mM sodium-phosphate buffer pH 7.8. The dialysed supernatant was applied to DEAE-Sephadex A-50 ion exchange column and the bound material eluted in 0-300 mM sodium chloride gradient. The fractions were assayed for protein content and for CR2 by spectrophotometric measurement at 280 nm and Western blotting (Fig.4-8). The elution of CR2 was achieved at 120-150 mM sodium chloride. The CR2 containing fractions were pooled and concentrated and a small aliquot was subjected to SDS-PAGE on 8 % gel followed by Coomassie staining of the proteins (Fig.4-9). An approximate level of expression of CR2 by insect cells ranges from 200 to 400 µg/l of cell culture.

4.3.3. Glycosylation of the insect cell expressed CR2

CR2 is heavily glycosylated protein with 11 potential glycosylation sites. The carbohydrate structures on CR2 have been implicated necessary for interaction with CD23 (Pochon *et al.*, 1992; Aubry *et al.*, 1994) and therefore the ability of CR2 to bind CD23 will depend on the type of glycosylation of the host cell. The insect cells are capable of protein glycosylation but the type of carbohydrate structures may be significantly different from the animal cells. In order to confirm the glycosylation of soluble CR2 expressed in insect cells by baculovirus expression system the partially purified protein was subjected to SDS-PAGE and subsequently transferred to nitrocellulose membrane. The glycoproteins were detected by GlycoTrack reagent kit and as can be seen in Fig.4-10, CR2 is indeed glycosylated. The contribution of carbohydrates to the total molecular weight is at least about 10 kDa, as calculated from the apparent molecular weight of the protein in SDS-PAGE (120 kDa) and the expected molecular weight of the nonglycosylated peptide precursor (110 kDa).

4.3.4. Binding of recombinant CR2 to recombinant CD23 by ELISA

In this experiment two different forms of recombinant CD23 were used. Apart from the

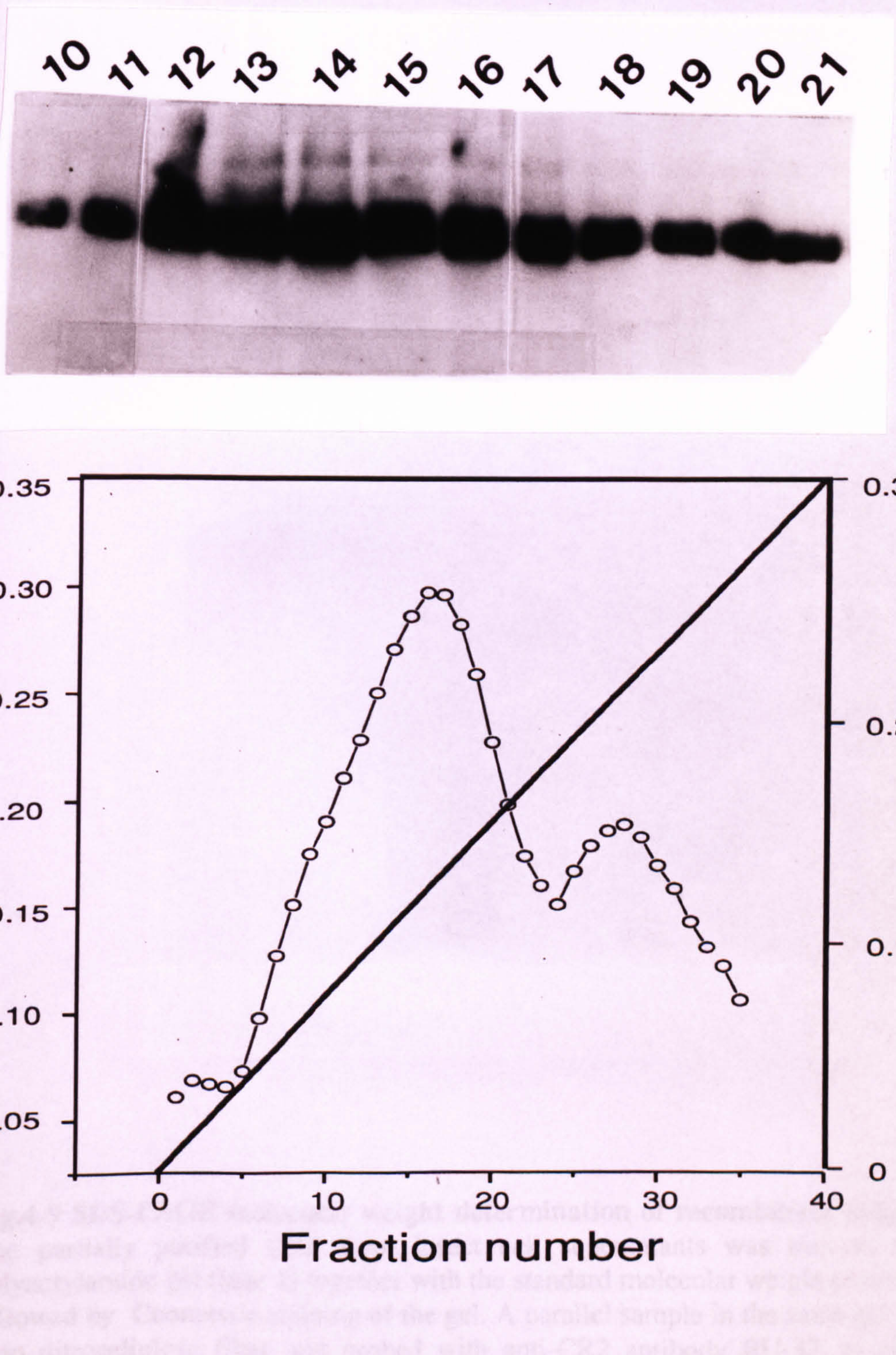


Fig.4-8 Purification of recombinant CR2 from insect cell supernatants by ion exchange on DEAE-Sephadex A-50 (Pharmacia).

The concentrated insect cell supernatant was dialysed against 10 mM sodium phosphate buffer pH 7.8 and applied on the DEAE-Sephadex A-50 column equilibrated with the same buffer. The bound protein was eluted in the continuous gradient of sodium chloride, 0-300 mM (prepared in the running buffer), and the fractions analyzed for protein content by spectrophotometry (graph) and for CR2 content by Western blotting (inserted photograph). CR2 was eluted over the range of 100-180 mM sodium chloride; the gradient of sodium chloride is indicated by the diagonal line.

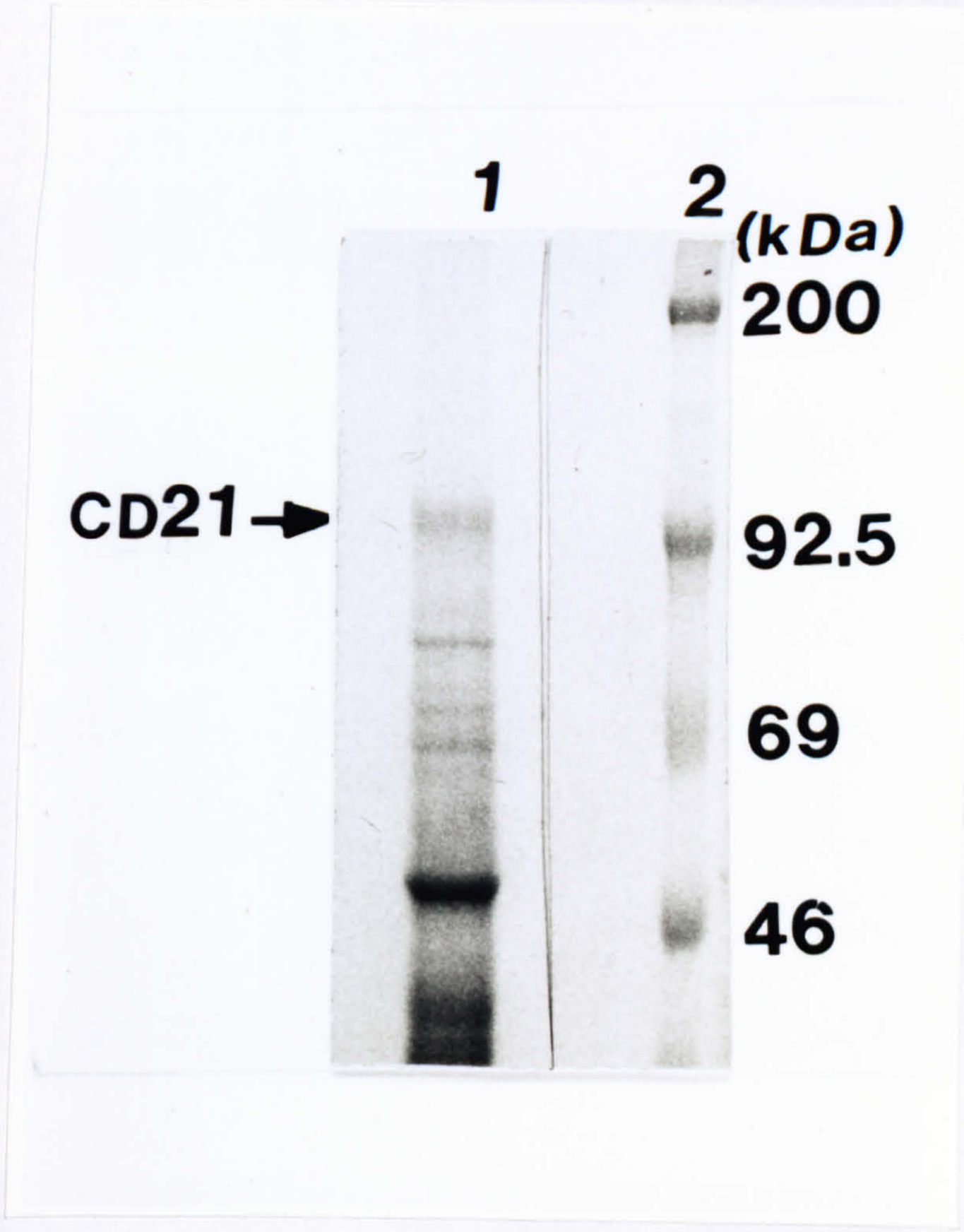


Fig.4-9 SDS-PAGE molecular weight determination of recombinant soluble CR2.
The partially purified CR2 from insect cell supernatants was run on 8 % SDS-polyacrylamide gel (lane 1) together with the standard molecular weight proteins (lane 2), followed by Coomassie staining of the gel. A parallel sample in the same gel was blotted onto nitrocellulose filter and probed with anti-CR2 antibody BU-32, to establish the position of CR2 in the gel (not shown). CR2 (CD21) migrates as a 120 kDa protein band (indicated by an arrow on the picture).

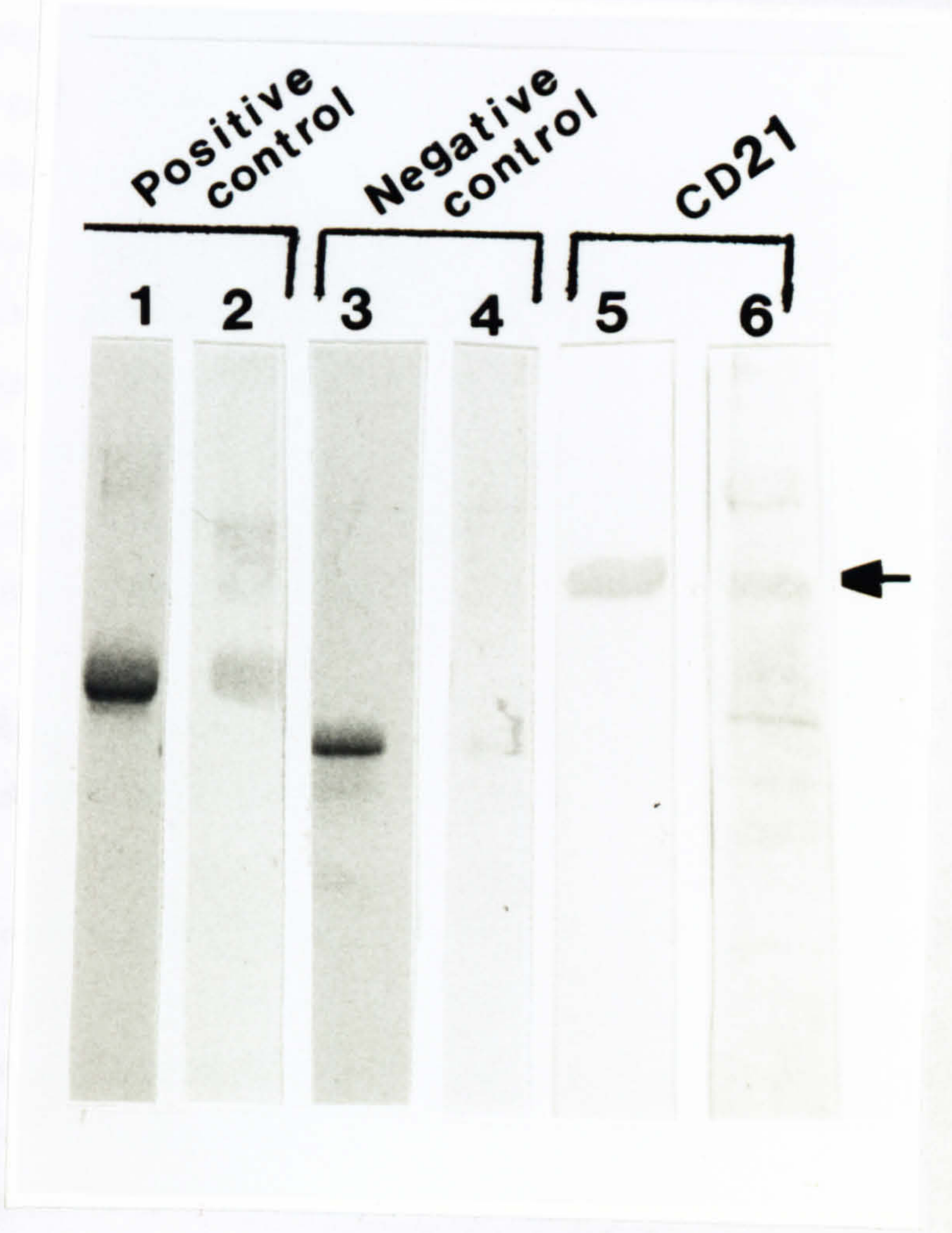


Fig.4-10 Glycosylation of recombinant CR2.
CR2 expressed in insect cells was analyzed for the presence of carbohydrates on the protein; a sample of CR2 together with the internal positive and negative controls for glycosylation of proteins (glycosylated CD23 expressed in NSO mammalian cell line and unglycosylated CD23 from *E. coli*, respectively) was probed with an antibody (BU32 for CR2, BU38 for CD23), (lanes 1, 3 and 5) or with the GlycoTrack commercial reagent for detection of carbohydrates (lanes 2, 4 and 6); the arrow indicates position of CR2; since in this segment of the gel there are no detectable contaminating proteins (Fig.4-9), the obtained signal can be ascribed to CR2, therefore confirming the glycosylated state of the insect cell expressed protein.

recombinant CD23 expressed in *E. coli* the other recombinant form was mammalian cell expressed soluble CD23 (J. Shi, PhD thesis), which differs from the bacterial protein for being glycosylated. CD23 was coupled to the ELISA plate at the increasing concentration 0-10 µg/ml and CR2 was added at the constant concentration of approximately 0.2 µg/ml. Following the incubation with CR2 an antibody to CR2 (BU-32) was added and, finally, secondary antibody peroxidase conjugate was added. All the incubation steps were carried out at room temperature for 1 h, followed by extensive washing of the unbound material. The negative control in the experiment was prepared in the exactly same way except that no CR2 was added. The result is shown in Fig.4-11. Both the *E. coli* and the mammalian cell expressed CD23 were able to bind the soluble CR2 from insect cells, with the bacterial protein binding somewhat better.

4.3.5. Binding of recombinant CD23 by the insect cell expressed CR2 immobilised on BIAcore sensor chip

In addition to confirming the interaction between the recombinant CD23 and CR2 one of the aims of this study was to measure the affinity constant of the interaction. The method of choice for this and other kinetic related studies was surface plasmon resonance (SPR). CR2 was immobilised on a BIAcore sensor chip and the recombinant CD23 was allowed to bind at different concentrations to the point of (near)saturation. After that the bound protein was allowed to dissociate from the immobilised receptor over a 15 min time period. In this way it was possible to measure the "on" (k_{+1}) and "off" (k_{-1}) rates of the interaction in real time and to calculate the affinity constant of the interaction. Fig.4-12 shows the sensograms obtained for recombinant CD23 from *E. coli* binding to immobilised CR2. A similar result with respect to the amount of bound protein was obtained for recombinant CD23 expressed in animal cells but a certain difference in the shape of the curve is notable. This refers to the alteration in the dissociation part of the curve as a consequence of a faster dissociation rate (Fig.4-13).

4.3.6. The affinity constant of CD23-CR2 interaction measured by SPR

The affinity constant of the interaction between the recombinant CD23 from *E. coli* and

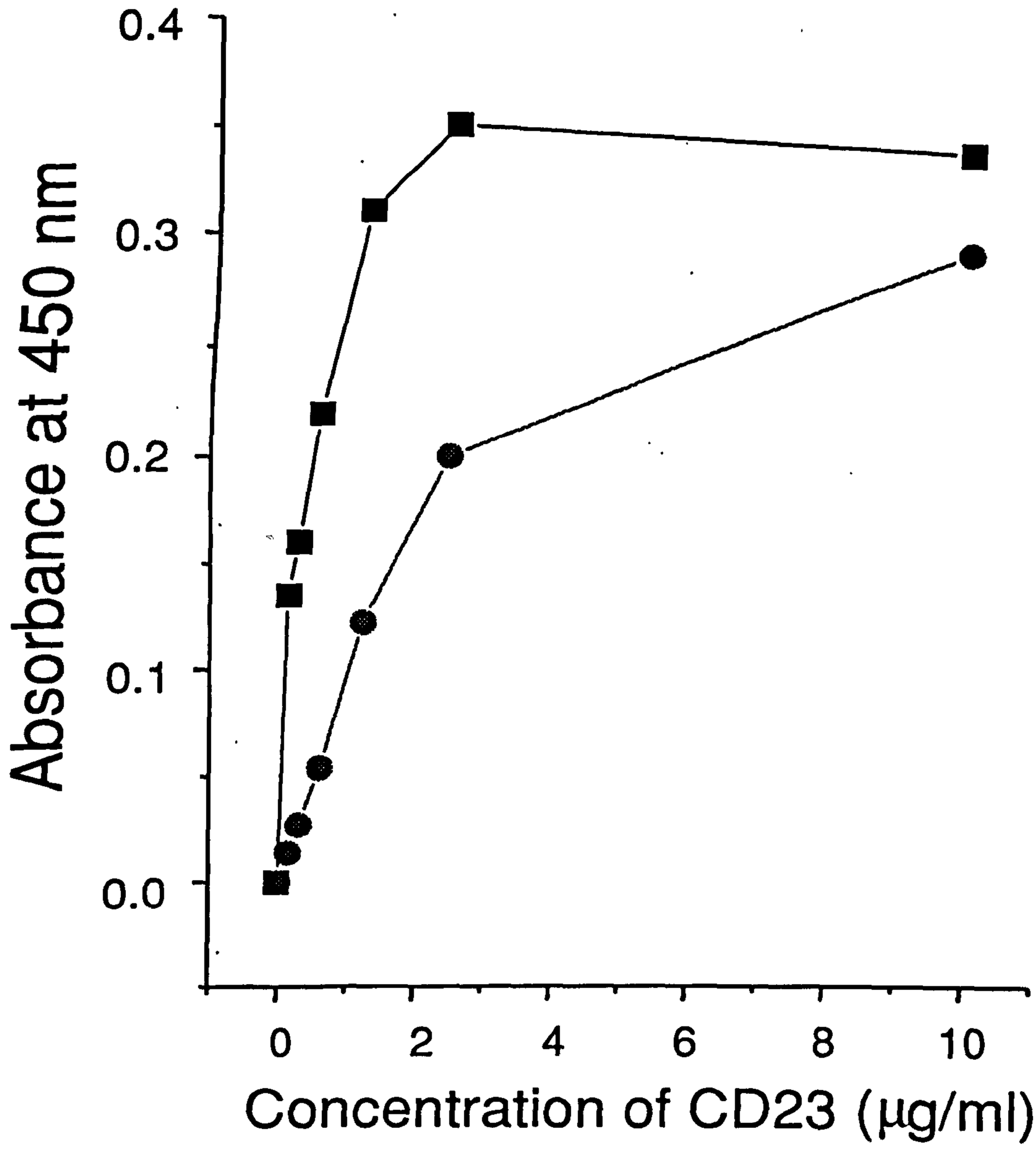


Fig.4-11 Binding of recombinant CR2 to recombinant CD23 by ELISA.
Recombinant CD23 from *E. coli* (squares), as well as the same CD23 fragment expressed in NSO mammalian cell line (circles) was coupled to the plastic plate and the recombinant soluble CR2 was added and the plates incubated for 1 h at the room temperature; after the removal of the unbound protein the bound CR2 was detected by an anti-CR2 antibody (BU32) and the secondary antibody peroxidase conjugate. The negative control in the experiment (no CR2 added) was subtracted from the readings.

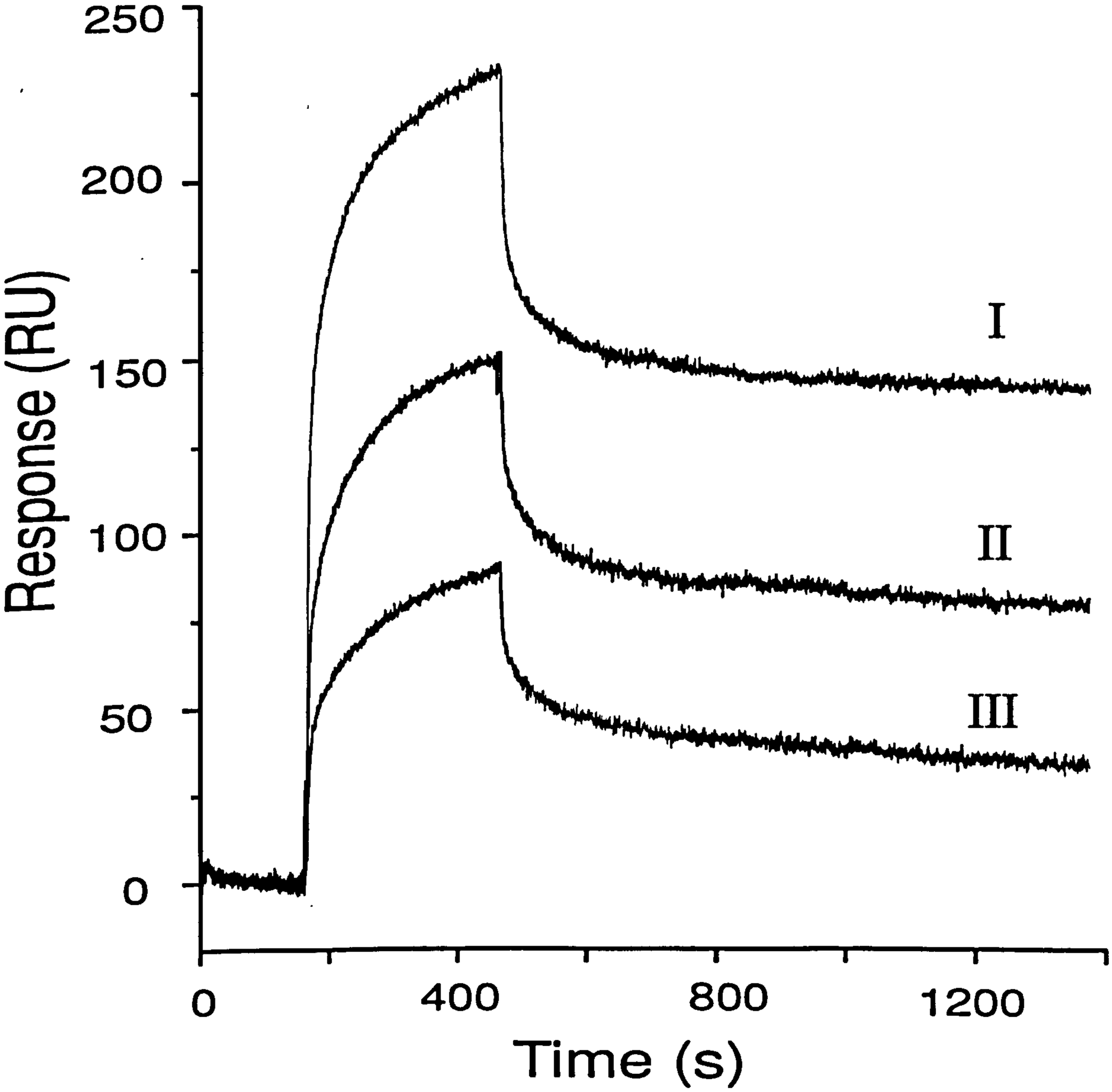


Fig.4-12 Binding of recombinant CD23 from *E. coli* to recombinant CR2 immobilised on BIAcore sensor chip.
CR2 was immobilised on the BIAcore chip and the recombinant CD23 from *E. coli* allowed to bind over 5 min; after that, the running buffer (HBS pH 7.2, 2 mM $CaCl_2$) was allowed to flow over the chip for approximately 15 min, followed by the removal of non-dissociated CD23 by two injections of 0.1 M glycine buffer pH 2.5. The curves represent sensograms obtained with the following concentrations of CD23: 300 (I), 200 (II) and 100 (III) µM, respectively. The response (ordinate axis) in this and all other SPR experiments is given in arbitrary resonance units.

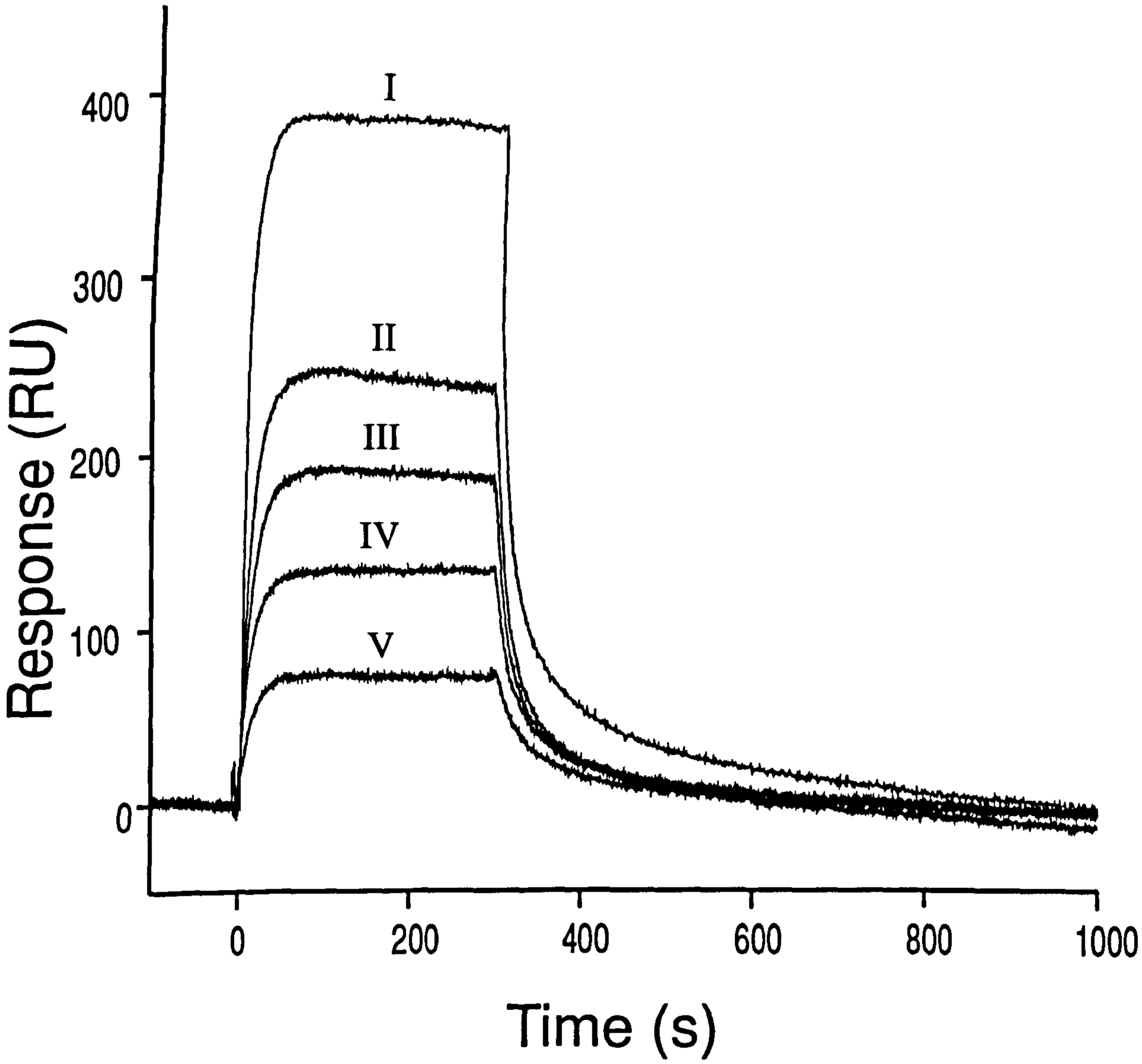


Fig.4-13 Binding of recombinant CD23 from NSO cell line to recombinant CR2 immobilised on BIAcore sensor chip.

The binding of mammalian cell expressed CD23 to recombinant CR2 from insect cells was performed in the exactly same way as for the CD23 from *E. coli* except that the injections of glycine buffer were not required, as the dissociation of the protein was complete after 15 min. The curves represent sensograms obtained with the following concentrations of CD23: 600 (I), 400 (II), 300 (III), 200 (IV) and 100 (V) µg/ml.

recombinant CR2 from insect cells was determined by SPR. The obtained values for association (k_{+1}) and dissociation (k_{-1}) rates were: $k_{+1} = 1.2 \times 10^3\ M^{-1}s^{-1}$ (SD = 0.07×10^3; N = 3) and $k_{-1} = 2.6 \times 10^{-3}\ s^{-1}$ (SD = 0.52×10^{-3}, N = 3). The ratio k^{+1}/k^{-1} gives the affinity constant Ka of $4.6 \times 10^5\ M^{-1}$ (SD = 0.88×10^5; N = 3). In the case of recombinant CD23 from animal cells the obtained figures were: $k_{+1} = 3.19 \times 10^3\ M^{-1}s^{-1}$ (SD = 1.10×10^3; N = 4), $k_{-1} = 3.12 \times 10^{-2}\ s^{-1}$ (SD = 6×10^{-3}, N = 4) and Ka = $1.0 \times 10^5\ M^{-1}$ (SD = 0.36×10^5; N = 4). These results indicate approximately 5-fold higher affinity of the recombinant CD23 from E. coli compared to mammalian cell expressed protein, presumably differing only in carbohydrate moiety. Summary of the SPR data for CD23-CR2 interaction is shown in Table 4-1.

Table 4-1: Summary of the kinetic data obtained for the interaction of recombinant CD23 and CR2 by SPR.

Kinetic parameter	CD23 from *E. coli*	CD23 from NS0
k_{+1} ($M^{-1}s^{-1}$)	1.2×10^3	3.2×10^3
k_{-1} (s^{-1})	2.6×10^{-3}	3.1×10^{-3}
Ka (M^{-1})	4.6×10^5	1.0×10^5

4.4. Simultaneous binding of IgE and CR2 to oligomeric CD23

The hypothesis that oligomeric CD23 could crosslink IgE and CR2 on the cell membrane (Sutton and Gould, 1993) was tested in an experiment utilising recombinant CR2 from insect cells, a recombinant Fc fragment of IgE and recombinant CD23 from *E. coli*, which was shown in the previous Chapter to be at least a dimer with the possibility of a dynamic balance between the dimer and trimer. The dimeric structure of CD23 should enable it to bind both ligands simultaneously. The recombinant CD23 was bound to the immobilised CR2 on the biosensor chip and subsequently, at the point of near-saturation, a mixture of CD23 and IgE-Fc (in the molar ratio 1:4) was injected. It was necessary to add a mixture

of CD23 and IgE rather then IgE alone to prevent the dissociation of the CR2-bound CD23. The further increase in binding was observed (Fig.4-14) and this binding could be attributed either to additional binding of CD23 to CR2 (as the saturation appears to be incomplete) or binding of IgE to the bound CD23. After subtracting the curve obtained for additional binding of CD23 alone in the second injection (parallel experiment), the observed increase in binding during the second injection was largely unchanged and can be therefore ascribed to the binding of IgE. Similar result was obtained by ELISA assay in which IgE was coupled to the plate and the recombinant CD23 from *E. coli* or animal cell line NSO added, followed by the addition of recombinant CR2. The bound CR2 was detected by an antibody to CR2 (BU-32) as previously described. The obtained results are shown in Fig.4-15.

4.5. Summary and conclusions

The major aim of this work was to investigate the possibility that other forms of CD23 apart from the intact molecule could bind the newly discovered ligand, CR2 (CD21). Aubry *et al*, (1992; 1994) and Pochon *et al*, (1992), demonstrated that native, full length CD23 incorporated in liposomes was able to bind CR2 on some B cells and suggested the involvement of carbohydrate structures on CR2 in the interaction. No soluble form of CD23, native or recombinant, was reported to exhibit the same binding capacity for CR2. On the other hand, all the soluble fragments of CD23 generated by proteolytic cleavage are able to bind to IgE although with different affinity. Provided that the interaction of CD23 and CR2 is indeed of the lectin type, all the soluble fragments of CD23 retaining the lectin domain might be expected to bind CR2. The reason that no CR2 binding activity with soluble CD23 has yet been reported is probably the inadequacy of the applied methods with regard to the sensitivity of assay and concentration requirements due to lower affinity of the interaction as compared to the native, full length CD23. The experimental approach undertaken in this work overcomes both of the mentioned difficulties by applying several different techniques for detecting the interaction, including the highly sensitive BIAcore assay while the expression of CD23 in *E. coli* provided an excellent source of the recombinant protein to meet the necessary concentration requirements.

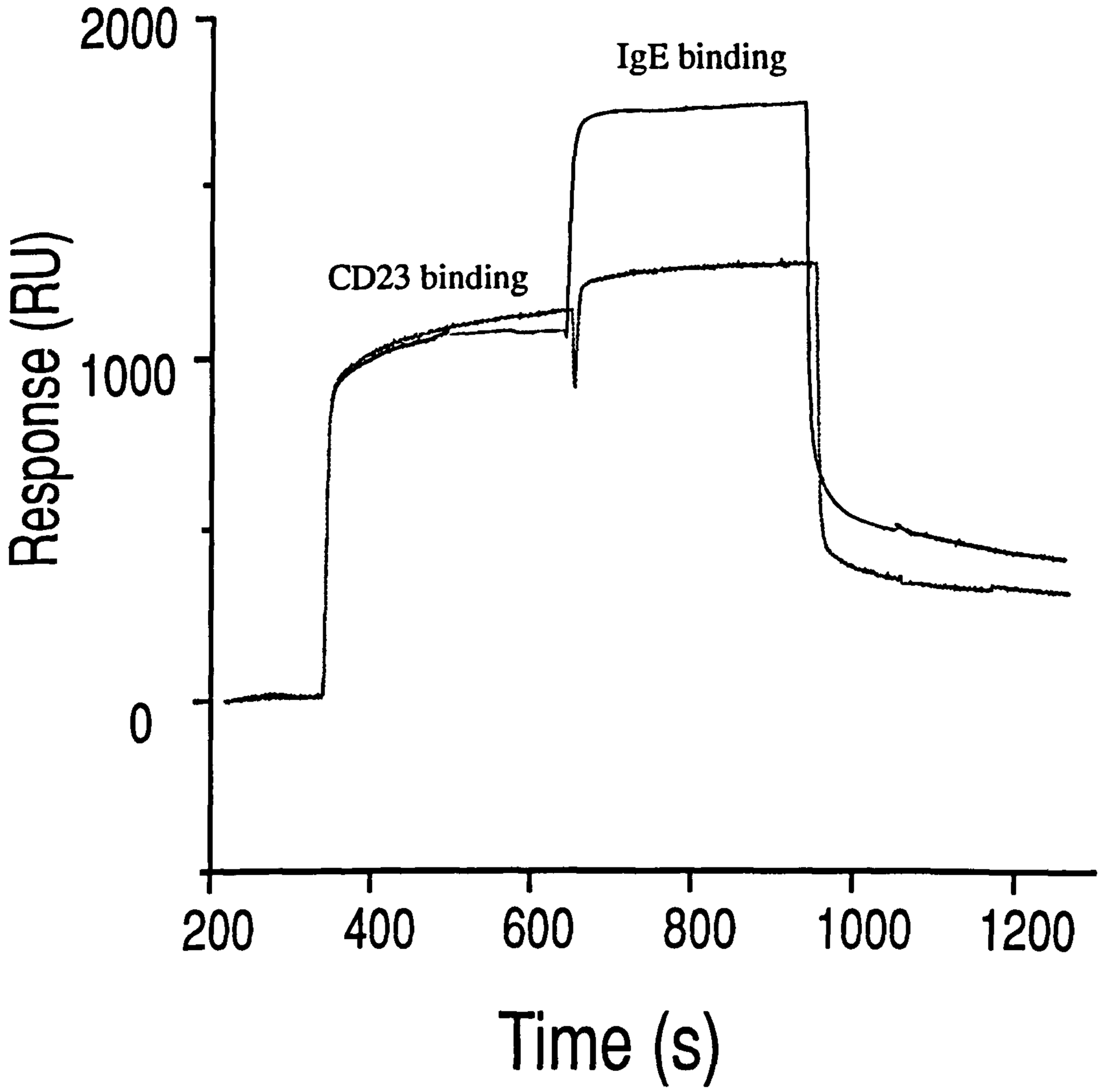


Fig.4-14 Two-step binding procedure for simultaneous binding of CR2 and IgE to CD23 by SPR.
In the first binding step CD23 from *E. coli* was allowed to bind to CR2 immobilised on biosensor chip and in the second binding step either CD23 alone was added at the same concentration as in the first binding step or in a mixture with IgE in the molar ratio 1:4. In a parallel experiment the same was done with a blank chip (negative control for binding in both cases). After appropriate substraction of negative curves the obtained resulting curves are shown above.

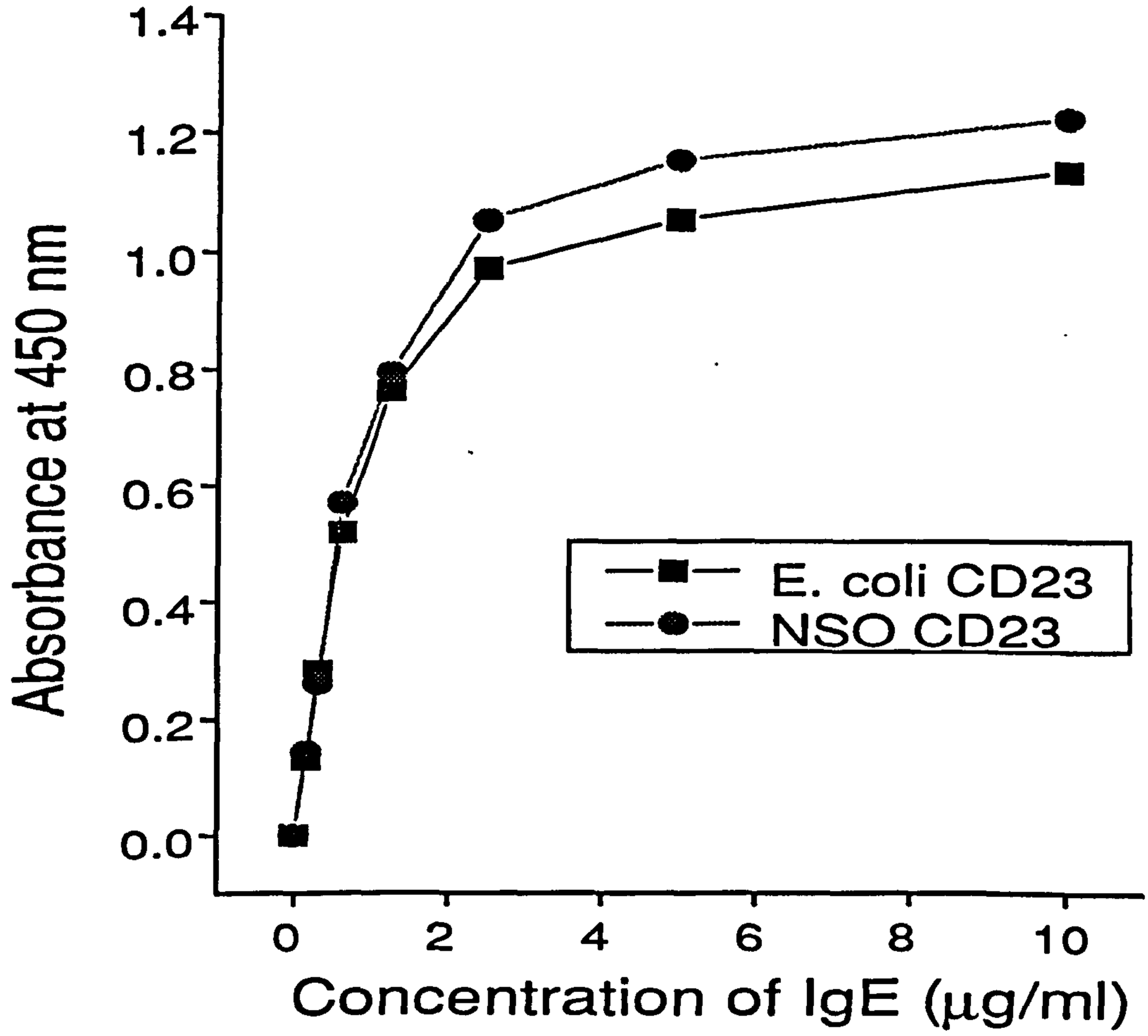


Fig.4-15 Simultaneous binding of CR2 and IgE to recombinant CD23 from *E. coli* in ELISA assay.

IgE was coupled to ELISA plate at different concentrations and the recombinant CD23 was added at saturating concentration (10 µg/ml). After that, recombinant CR2 was added followed by an anti-CR2 antibody (BU-32) and the secondary antibody peroxidase conjugate. The negative control in the experiment (no IgE) added was subtracted from the readings.

It was found that recombinant soluble CD23 does indeed bind the native CR2 on RPMI 8226 cells according to its capacity to inhibit completely the binding of CD23-liposomes to these cells. It was also found that recombinant CR2 expressed in insect cells binds recombinant CD23 from *E. coli* and NSO cell line. However, the binding in the latter case appears to be rather weak because it could not be detected in a cell binding assay in which the soluble CR2 was added to the suspension of CD23 positive cells, followed by anti-CR2 antibody addition and FACs analysis (not shown), while the highly sensitive SPR method gave a reproducible result for binding although with what may be considered a relatively low affinity (the affinity constant was in the range 10^{-5}-10^{-6} M^{-1}, which is approximately one order of magnitude less than Ka obtained for binding of the same CD23 preparations to IgE). If the carbohydrate structures on CR2 are indeed critically involved into the interaction with CD23 then the apparently low affinity constant may be explained by possibly inappropriate or insufficient glycosylation of CR2 in insect cells; in that case, the observed binding would confirm the prediction that some basal binding does indeed take place, regardless of the cell type dependent glycosylation, as a consequence of the contribution of the peptide structures of SCRs 1 and 2 to the binding (Aubry *et al.*, 1994). This interpretation, however, should be considered with a due allowance for the possibility of CD23 having wider carbohydrate binding specificity and thus being able to bind the altered (possibly only partially) carbohydrate structures on recombinant CR2 with a lower affinity. It is difficult to distinguish with certainty between the two possibilities. An attempt had been made in this work to measure the affinity constant of CD23 for native CR2 but the insufficient amount of the protein and the interference of the used detergent, posed great obstacles in completing this task. This work is still in progress, but the current work, however, does demonstrate that the requirements for the interaction between CD23 and CR2 may not be as strict as it was initially thought, regarding the structure and glycosylation of CR2.

Further to that, the evidence that soluble CD23 can crosslink IgE and CR2 as proposed by Sutton and Gould, 1993, has been presented. The dimeric recombinant CD23 from *E. coli* bound to the immobilised CR2 on the BIAcore sensor chip was able to bind IgE (Fc fragment) which indicates that oligomeric CD23 can indeed interact simultaneously with both ligands, at least *in vitro*. Further work is currently in progress to confirm this

observation *in vivo* conditions and the B cell line co-expressing CR2 and IgE has been already made. Therefore, it will be possible to look at the functional consequence of the crosslinking of IgE and CR2 on cell membrane by soluble CD23 fragments.

CHAPTER 5: CD23 IS A COSTIMULATOR OF B CELL ACTIVATION

5.1. Introduction

5.1.1. The B cells differentiation pathway

The B lymphocytes are responsible for fighting infections by producing specific antibodies that recognise and bind to the target antigen. To achieve this function the B cells have to undergo a rather complex pathway including several distinct stages of development. B lymphocytes are generated from pluripotent stem cells in the bone marrow via a number of precursor B cell phenotypes leading to the rise of cells that express on their surface immunoglobulin M (IgM). If challenged by an antigen at this stage of maturation these cells undergo clonal deletion accomplished by programmed cell death (apoptosis), the biological significance of which is the removal of those B cells that express antigen receptors that bind self tissue components and may potentially lead to autoimmune disease. The immature B cells develops further to express membrane IgD in addition to already present IgM and these "mature" B cells can now respond positively to the challenge of an antigen. The antigen-dependent differentiation of B cells is initiated by the ligation of the antigen receptor (membrane IgM, mIgM) and gives rise to either high rate IgM antibody secreting plasma cells or antigen specific memory B cells, which have undergone immunoglobulin isotype switching. In the latter case two cellular events are involved, follicular reaction and germinal centre formation (Liu *et al.*, 1992). Follicles are found in secondary lymphoid tissues such as tonsils, spleen and lymph nodes and consist of a network of follicular dendritic cells (FDC) and large numbers of lymphocytes. The FDCs can immobilise antigen and present it to the memory B cells which in turn begin to proliferate. Once the network is filled with the newly formed cells, polarisation of cells within the follicle occurs giving rise to the typical histological appearance of germinal center. The further proliferation of the cells is accompanied by the loss of membrane Ig expression and migration towards the mantle zone of the follicle, finally, giving rise to non-dividing centrocytes which express membrane Ig other than IgM. The centrocytes normally undergo the programmed cell death pathway unless

challenged by antigen that had initiated their formation or rescued by certain cellular signals that are only beginning to be understood. One of the two known signals required for the rescue of centrocytes from apoptosis is the ligation of CD40 (Liu *et al.*, 1989) and the other is stimulation with soluble CD23 and interleukin-1α/IL-1α (Liu et al., 1991; 1992). The antigen specific selection of the centrocytes leads to the final stage of B cell development, i.e. generation of IgG, IgA or IgE antibody secreting plasma cells.

In the absence of an antigen the activation of mature B cells can be induced by anti-IgM antibodies, providing a good model for studying the cellular response *in vitro*. Numerous studies have shown that addition of monoclonal antibodies against IgM to the resting B cells stimulates them to proliferate, as observed by both calcium mobilization and DNA synthesis. IgM alone is not capable of transducing the signals but does so through association with several other membrane proteins, notably the tightly associated Ig-α and Ig-β chains (Hombach et al. 1990), and the more loosely associated CD19/CR2/TAPA-1 complex (Fearon and Carter, 1995). The association of IgM with the accessory molecules Ig-α and Ig-β has led some researchers to introduce the term "antigen receptor complex" (Cushley and Harnett, 1993). Both the associated Ig chains and the CD19/CR2/TAPA-1 complex provide the antigen receptor with the necessary signal-transducing elements and in addition to that, the latter amplifies the response of the B cell to the ligation of antigen receptor. Several reports have shown that crosslinking of CR2 or CD19 to CD32 (the low affinity receptor for IgG, FcγRII) with monoclonal antibodies results in the amplification of B cell proliferation (Carter et al., 1988; Carter and Fearon, 1992; Sato et al., 1995). In addition to enhancing the IgM response upon ligation, CD19 also exhibits a unique capacity to decrease the threshold of anti-IgM antibody concentration that is required to initiate cell differentiation, once it is crosslinked to CD32 via monoclonal anti-CD19 antibody (Carter and Fearon, 1992). Soluble antibodies to either CD19 or CR2 suppress rather than enhance IgM-mediated B cell proliferation. In contrast to CD19 and CR2, another B cell antigen, CD40, was shown capable of stimulating cell proliferation independently of IgM (Banchereau et al., 1990). Again, the activation was possible only when CD40 was crosslinked to CD32 on a fibroblast cell line *via* an anti-CD40 monoclonal antibody.

5.1.2. CD23 and B cell activation

The role of CD23 in B cell activation began to emerge with the discovery that this membrane protein is also B cell differentiation marker, in addition to being the low affinity receptor for IgE. As mentioned before, soluble CD23 displays autocrine growth factor activity (Gordon *et al.*, 1991; Conrad, 1990; Delespesse *et al.*, 1989) and is also reported to stimulate the differentiation of centrocytes from B cell germinal centers *in vitro* (Liu *et al.*, 1991). Bonnefoy *et al.*, (1993), demonstrated that a subset of anti-CR2 antibodies, as well as soluble CD23 in conjunction with IL-1α, can promote the rescue of germinal center B cells from apoptosis, indicating that the interaction of soluble CD23 with CR2, which is expressed on B centrocytes, is probably responsible for this effect. FDCs also express membrane associated CD23 at a high level and, in contrast to soluble CD23, no suggestion has been made yet as to its role in B cell activation. Sutton and Gould (1993) have proposed a model in which the membrane CD23 may interact synergistically with the low affinity receptor for IgG, FcγRII/CD32,on antigen presenting cells, the consequence of which would be either the enhancement of the IgM-mediated B cell response to antigen or lowering the threshold of the antigen concentration required for the activation of the cell. This hypothesis is based on the analogy to the synergistic interaction of IgM and CD19 after ligation of both receptors, in which case it was shown that in addition to the enhanced response of the cell a 100-fold lower concentration of anti-IgM antibody was sufficient to stimulate cell proliferation after crosslinking IgM and CD19, as compared to IgM alone. In order to subject this model to an experimental test, highly purified resting B cells from human tonsils were isolated and co-incubated with a fibroblast cell line which has been transformed with both CD23 and CD32 in the presence of anti-IgM antibody at a wide range of concentration (10^{-15}-10^{-7} M). This Chapter presents the evidence on the effect of membrane associated CD23 on B cell activation and discusses the role of CD23-CR2 interaction in the context of FDC - B cell interactions.

5.2. Generation of fibroblast cell line expressing CD23 and CD32

5.2.1. Construction of the expression vector pML95

The expression vector pML95 (Fig.5-1) was made from the plasmid pEE12 (R.J. Owens, Celltech Ltd., Slough, UK) to which the full length CD23 cDNA in the form of a *Hind III-EcoRI* fragment was inserted, as described in section 2.2.3. This plasmid contains the hCMV (human cytomegalovirus) promoter and glutamine synthetase gene (GS) for selection of transformants in animal cells, in addition to ampicillin resistance gene for propagation in bacteria.

5.2.2. Transfection of mouse fibroblast cells with pML95 vector

The neomycin resistant mouse fibroblast cell line expressing human FcγRII was transfected with the plasmid pML95 by the method of lipofection. Following transfection, the cells were incubated in low glutamine selection medium for 3 weeks in the presence of different concentrations of MSX (methionine sulphoximine), with no success in selecting the transformed cells. This was due to the high level expression of the endogenous glutamine synthetase gene, which has made it difficult to kill non-transformed cells. Therefore, an alternative approach was undertaken in which the expression vector pML95 was co-transfected with another plasmid (pRTK3) carrying the gene for thymidine kinase and the cells selected for their ability to grow in HAT medium. Following the selection in HAT medium the transformants were routinely grown in HT medium containing 1 mg/ml G418.

5.2.3. Expression of CD23 in fibroblast cells

To confirm the expression of CD23 on the cell surface the cells were immuno-stained with BU-38 antibody and analyzed for fluorescence by flow cytometry (FACs). As can be seen on Fig.5-2 the fibroblast cells transformed with pML95 plasmid do express CD23 on the cell surface. It is also clear from this figure that only about 50 % of cells selected for their ability to grow in HAT medium are in fact CD23 positive. To obtain

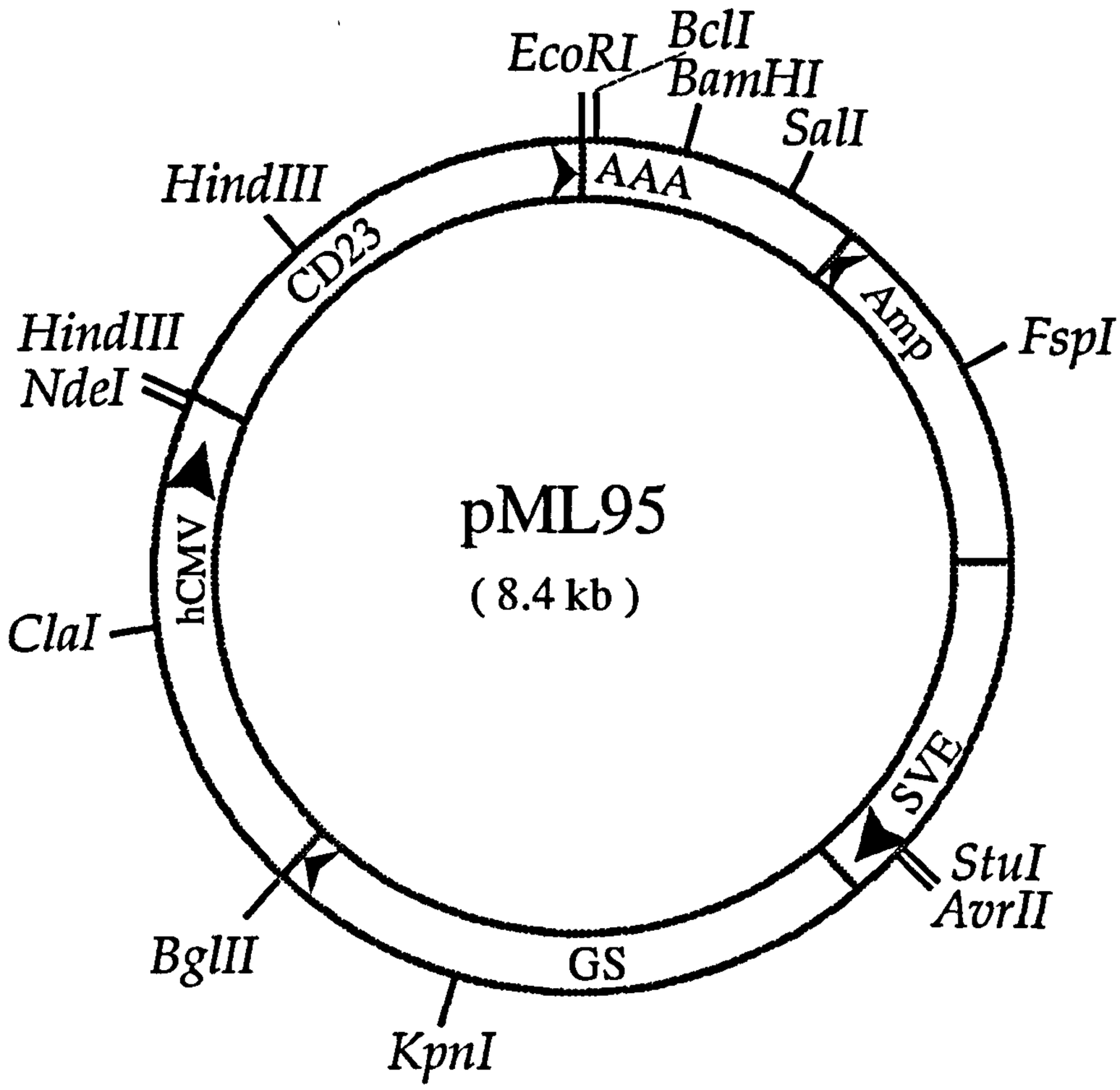


Fig.5-1 pML95 vector for expression of full length human CD23 in mammalian cells. This vector is based on the plasmid pEE12 (Celltech) to which the cDNA for human CD23 was inserted as a *HindIII-EcoRI* fragment (partial digestion with *HindIII* of the CD23 cDNA donor plasmid pUC-18 was required to preserve the *HindIII* site within the insert).The plasmid contains the human cytomegalovirus promoter (hCMV), the poly-A signal for termination of the transcription, the ampicillin resistance gene for propagation in bacteria and the glutamine synthetase gene for selection in mammalian cells (GS). The total size of the plasmid is 8.4 kb and the plasmid can be also used in cotransfection experiments.

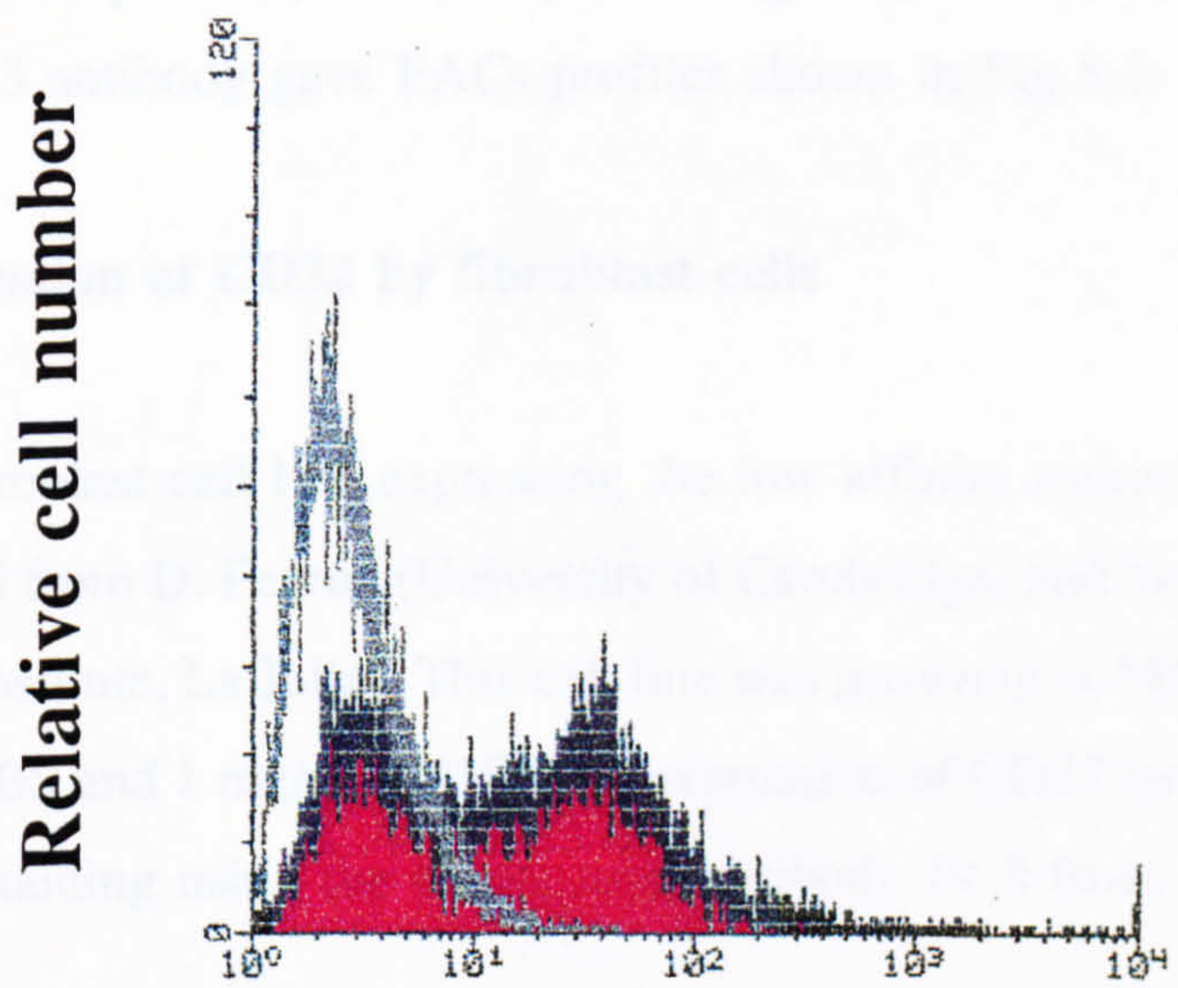


Fig.5-2 Expression of CD23 in fibroblast cells.
The TK⁻ fibroblast cells that already expressed CD32 were cotransfected with plasmids pML95 (having the CD23 cDNA insert) and pRTK3 (containing the thymidine kinase gene) by the lipofection method and the cells were selected for their ability to survive and grow in HAT medium. The FACs profile shown above is obtained by staining the fibroblast cells with the anti-CD23 antibody BU-38 and the negative control represents the cells staining by an isotype matched negative IgG.

homogenous cell population either positive or negative for CD23 expression, single clones from the initial population were isolated by the method of limited dilution and expanded. Among the several clones tested two were selected for all future studies. These were called F6 (CD23 positive) and C11 (CD23 negative). The staining of these cells with BU-38 anti-CD23 antibody gave FACs profiles shown in Fig.5-3.

5.2.4. Expression of CD32 by fibroblast cells

The Ltk$^-$ fibroblast cell line expressing the low affinity receptor for IgG (FcγRII/CD32) was obtained from D. Fearon (University of Cambridge) and has been made by J. Ravetch (SCRIPPS Institute, La Jolla). This cell line was grown in α-MEM medium supplemented with 10 % FBS and 1 mg/ml G418. The expression of CD32 by these cells was confirmed by immunostaining using the monoclonal antibody IV.3 from Medarex (Fig.5-4).

5.3. Purification of mature B cells from human tonsils

The human tonsils from children undergoing routine tonsillectomy were obtained 30 min - 2 h after the surgery and were used for isolation of B lymphocytes immediately. Following the dissection of the tissue the cells were released by gentle teasing against the surface of the container so that the release of dendritic cell was minimal. The total number of cells released from one single pair of human tonsils was typically about 1×10^9 and the number of purified B cells at the end of the procedure was about 5×10^7. The results obtained for a representative experiment are summarised in Table 5-1. The cells were examined for phenotypic expression of the surface antigens to confirm the B lineage and purity, as well as to define stage of development. The FACs profiles obtained for staining of the cells with different antibodies are shown in Fig.5-5. The staining of cells with anti-CD19 antibody HD37 shows that almost the entire cell population (> 95 %) consisted of B cells, while the cells did not stain with either the OKT3 antibody specific for CD3 (T lymphocytes) or with an anti-CD16 antibody (NK cells and granulocytes). The cells were largely IgM positive and also showed partial staining for the CD21 and CD23 antigens. The cell number did not increase over 24-48 h incubation period following the isolation and in fact it decreased as the result of spontaneous apoptosis of the germinal

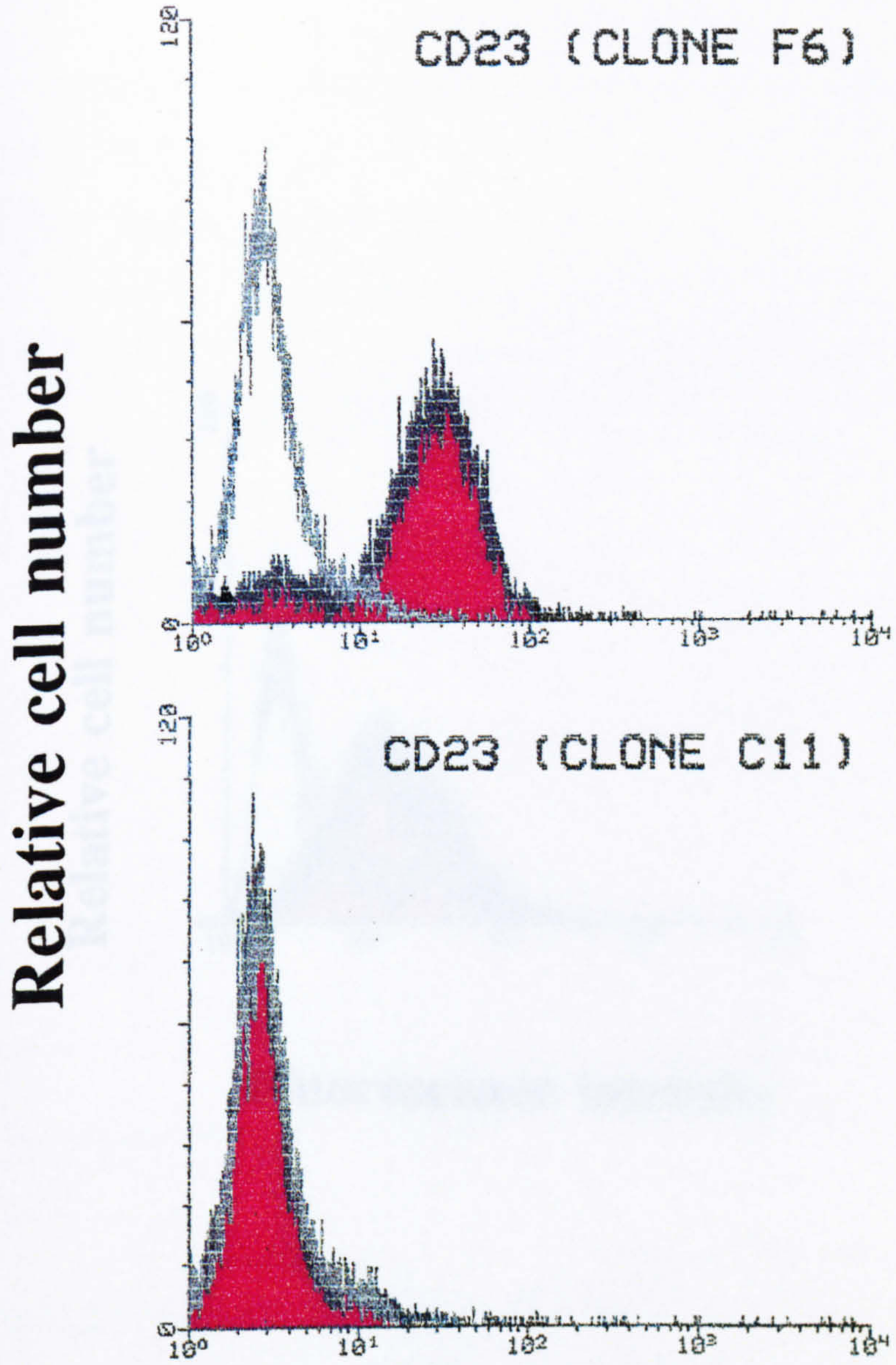


Fig.5-3 Selection and isolation of single CD23$^+$ and CD23$^-$ clones from the transformed fibroblast cells.

Staining of the cells with BU-38 anti-CD23 was performed following the HAT selection, isolation of single clones by the method of limited dilution and expansion of these clones in HT medium. Two clones, one of which was CD23 positive (termed F6) and the other CD23 negative (termed C11) were used throughout the study.

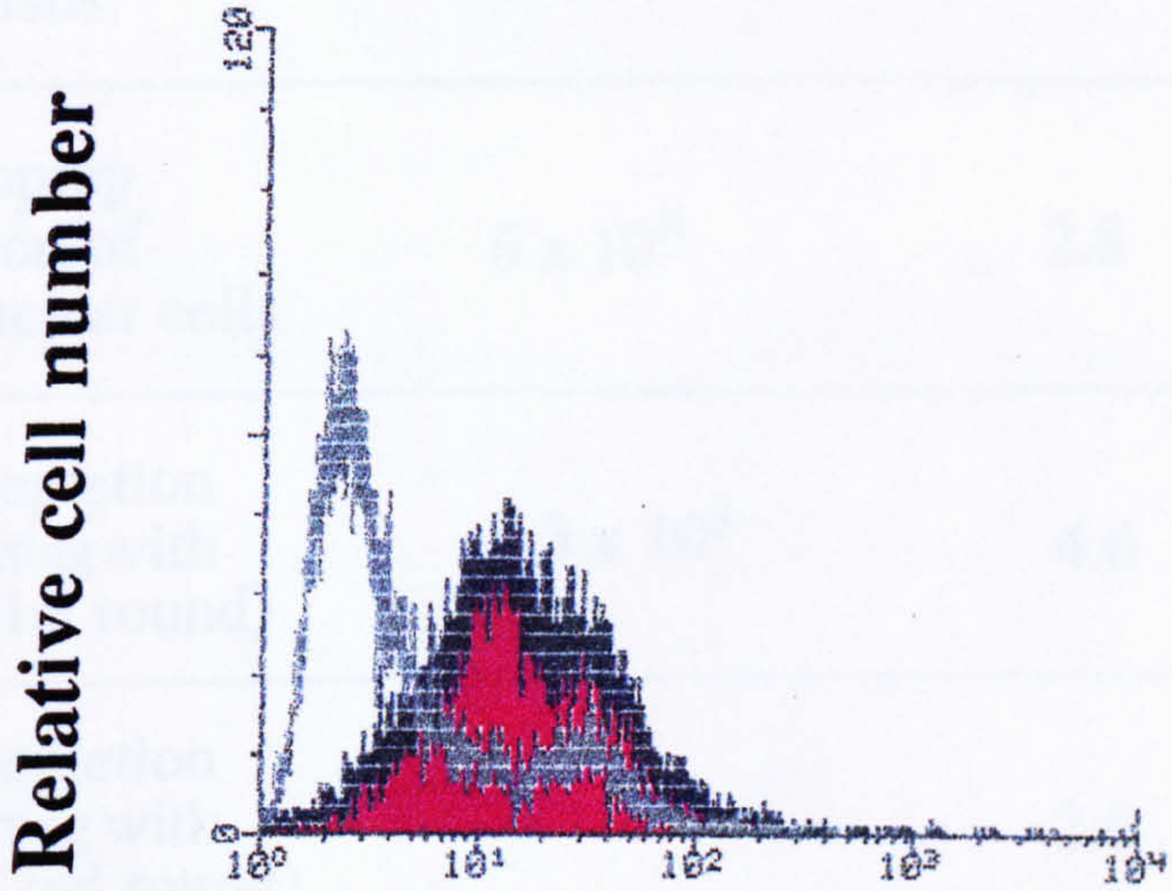


Fig.5-4 Expression of CD32 in fibroblast cells.
The fibroblast cells were stained with anti-CD32 antibody IV.3 followed by the secondary antibody FITC conjugate and analyzed by FACs. The negative control for staining was an isotype matched negative antibody (represented by the open peak on the graph).

Table 5-1: Isolation and purification of resting B cells from human tonsils.

Purification stage	Total cells	Purification factor	Yield (%)
Release of cells from tonsils	1.7×10^9	1	100
Lymphoprep separation of mononuclear cells	6×10^8	2.8	35.3
T cell depletion by rosetting with SRBC (1st round)	1.3×10^8	4.6	7.6
T cell depletion by rosetting with SRBC (2nd round)	5×10^7	2.6	2.9
Overnight incubation	4×10^7	1.2	2.4
Percoll gradient	1.8×10^7	2.2	1.1

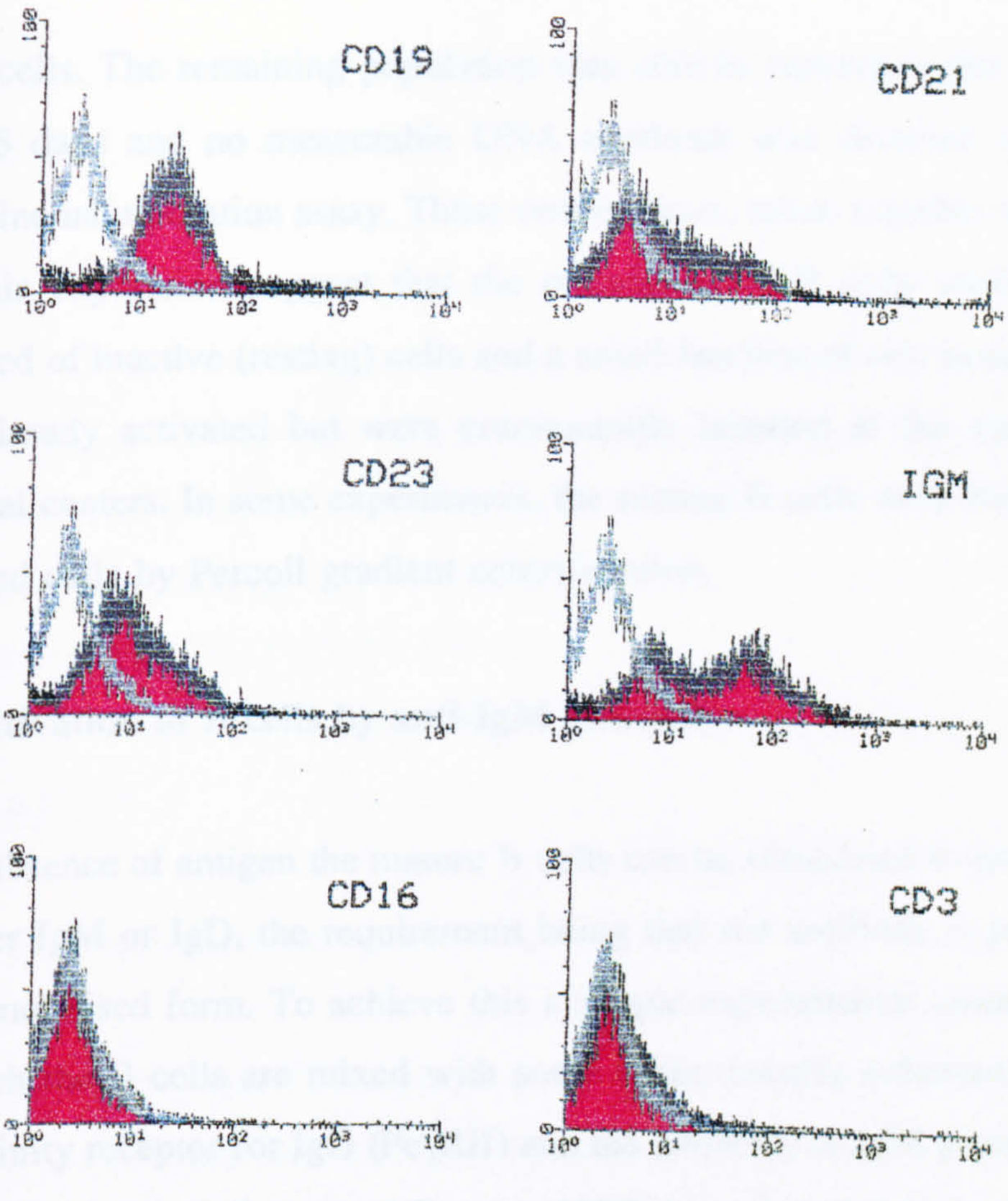


Fig.5-5 Phenotypic characterisation of tonsillar B cells.
Following the isolation and purification of the B cells from human tonsils the cells were stained for several different surface antigens to confirm the B lineage and purity. More than 95 % cells were CD19 positive and 80 % were also IgM positive. No detectable staining was observed for CD3 (T cell marker) and CD16 (NK cells and granulocytes). The antibodies used were HD37 (CD19), BU-36 (CD21), BU-38 (CD23), DAA.4 (IgM), Dako-CD16 (CD16) and OKT3 (CD3).

center cells. The remaining population was able to survive in the culture conditions for up to 5 days and no measurable DNA synthesis was detected according to the [^{3}H]-thymidine incorporation assay. These observations, taken together with the FACs data for antigenic expression suggest that the population of B cells used in this work largely consisted of inactive (resting) cells and a small fraction of non-proliferating cells that had been already activated but were consequently arrested at the stage prior entering the germinal centers. In some experiments, the resting B cells were further purified from the activated cells by Percoll gradient centrifugation.

5.4. Activation of B cells by anti-IgM antibody

In the absence of antigen the mature B cells can be stimulated to proliferate by antibodies to either IgM or IgD, the requirement being that the antibody is presented to the cell in the immobilised form. To achieve this a simple experimental model has been developed in which the B cells are mixed with some other, usually adherent,cells that express the low affinity receptor for IgG (FcγRII) and the antibody to IgM presented to B cells in this crosslinked fashion. Carter and Fearon (1992) have found that the minimal concentration of anti-IgM antibody DA4.4 required to stimulate the cell was 6.7×10^{-11} M but the optimal concentration for maximum stimulation of DNA synthesis was 10^{-8}-10^{-7} M. In this work a range of anti-IgM concentration from 10^{-15} - 10^{-7} M was applied in order to find out the minimal required concentration of the antibody that is still able to activate the cell, as well as to determine the maximal concentration at which the antibody can be presented to the B cells by FcγRII bearing fibroblast cells so that no free antibody which could inhibit stimulation remains in the solution. It was found that minimal required concentration of the antibody to activate the cells was similar to that one reported by Carter and Fearon (1992) and was in the range 10^{-11}-10^{-10} M. The background incorporation was about 2000-3000 cpm. The concentration of antibody at which the maximum stimulation was achieved was 10^{-8} M and further increase caused inhibition. This is shown throughout figures 5-5 to 5-9 which are detailed below.

5.5. Co-ligation of CD23 and CD32 increases the responsiveness of B cell to IgM ligation by factor 10-100.

Tonsillar B cells were incubated with mitomycin treated either $CD32^+/CD23^-$ or $CD32^+/CD23^+$ fibroblasts in the presence of incremental concentrations of anti-IgM and 200 U/ml of IL-4. Following 48 h incubation the cells were labelled with [^{3}H]-thymidine for 12-16 h, harvested on fibre glass paper and the incorporation of the label measured by scintillation counting. The minimal concentration of anti-IgM that stimulated the cells in the presence of $CD32^+/CD23^-$ fibroblasts was 10^{-11} M; in the presence of $CD32^+/CD23^+$ cells it was reduced by a factor 10-100. Two representative experiments are shown in Fig.5-6 and 5-8.

5.6. CD23 is mitogenic for pre-activated B cells

Fig.5-6 also shows that CD23 enhances the cellular response to antigen receptor ligation which is particularly obvious for the suboptimal concentrations of anti-IgM. The stimulatory activity of CD23 is even more noticeable in the absence of IL-4, although no change of the threshold concentration of anti-IgM required for the cell activation could be observed in this case (Fig.5-7). It therefore appears that CD23 can simulate the cytokine activity of IL-4 but does not synergise with it.

5.7. Recombinant CR2 inhibits the stimulatory activity of CD23

In order to test if CR2 is the counterstructure for CD23 on B cells the recombinant CR2 expressed in insect cells and partially purified by DEAE-Sephadex A50 ion exchange chromatography was added to the medium containing B cells and fibroblasts and the appropriate stimuli. The incubation conditions were exactly as above. As can be seen from Fig.5-8 the B cell stimulatory activity of CD23 is completely inhibited in the presence of recombinant soluble CR2. Note that CR2 caused an additional slight inhibition of the IgM mediated B cell activation.

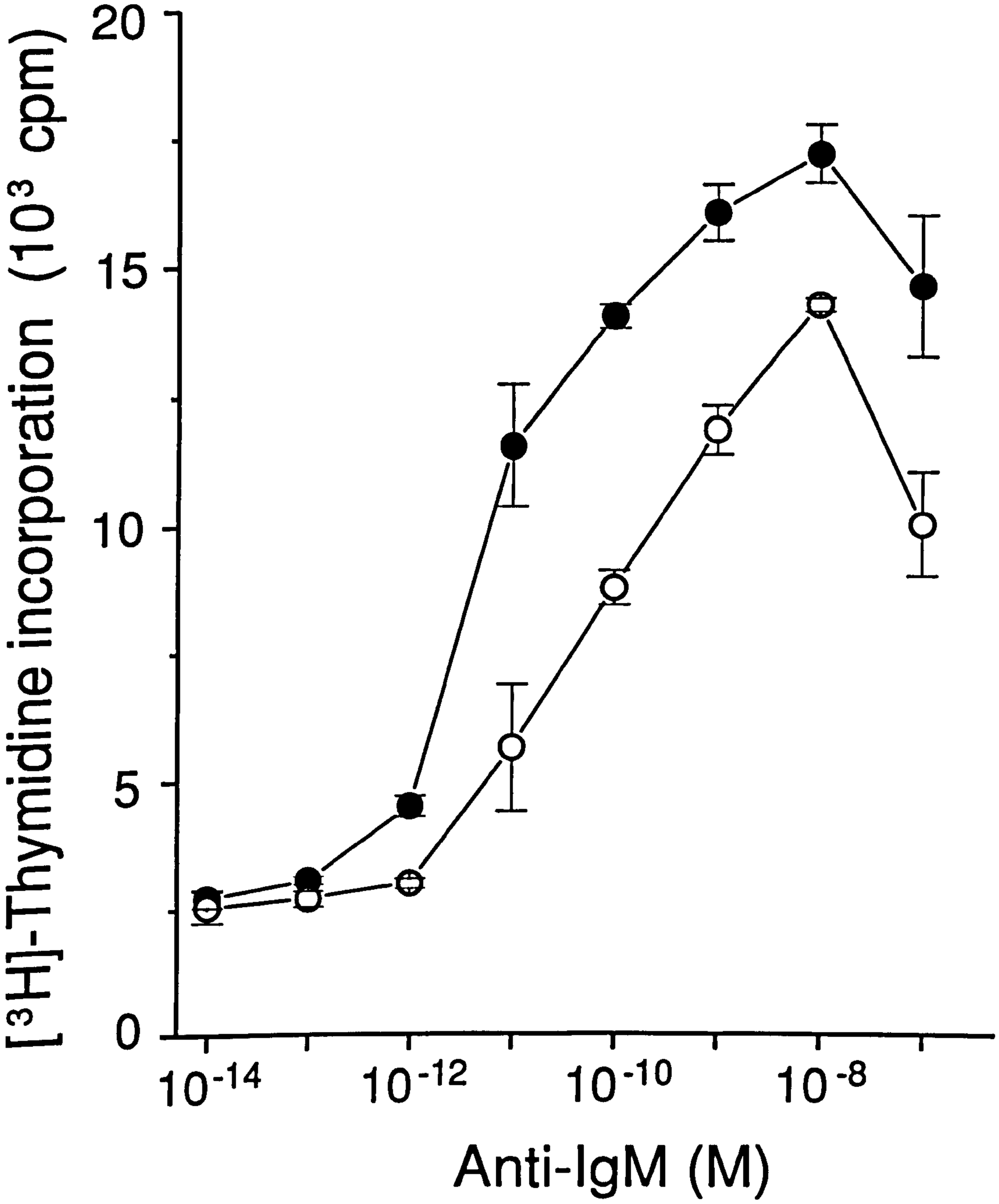


Fig.5-6 CD23 is a costimulator of B cell activation.
Triplicate samples of 5 x 10^4 tonsillar B cells were cultured together with 2 x 10^4 mitomycin treated $CD32^+/CD23^-$ (○) or $CD32^+/CD23^+$ (●) cells in 96-well tissue culture plates, in the presence of 200 U/ml of IL-4 and the incremental concentrations of anti-IgM. Following 48 h incubation, the cells were labelled with 1 μCi of [^{3}H]-Thymidine for 16 h. The results in this and all following experiments in this Chapter are shown as the means of triplicates, +/- SE (standard error). The background incorporation of the label was about 2500 cpm.

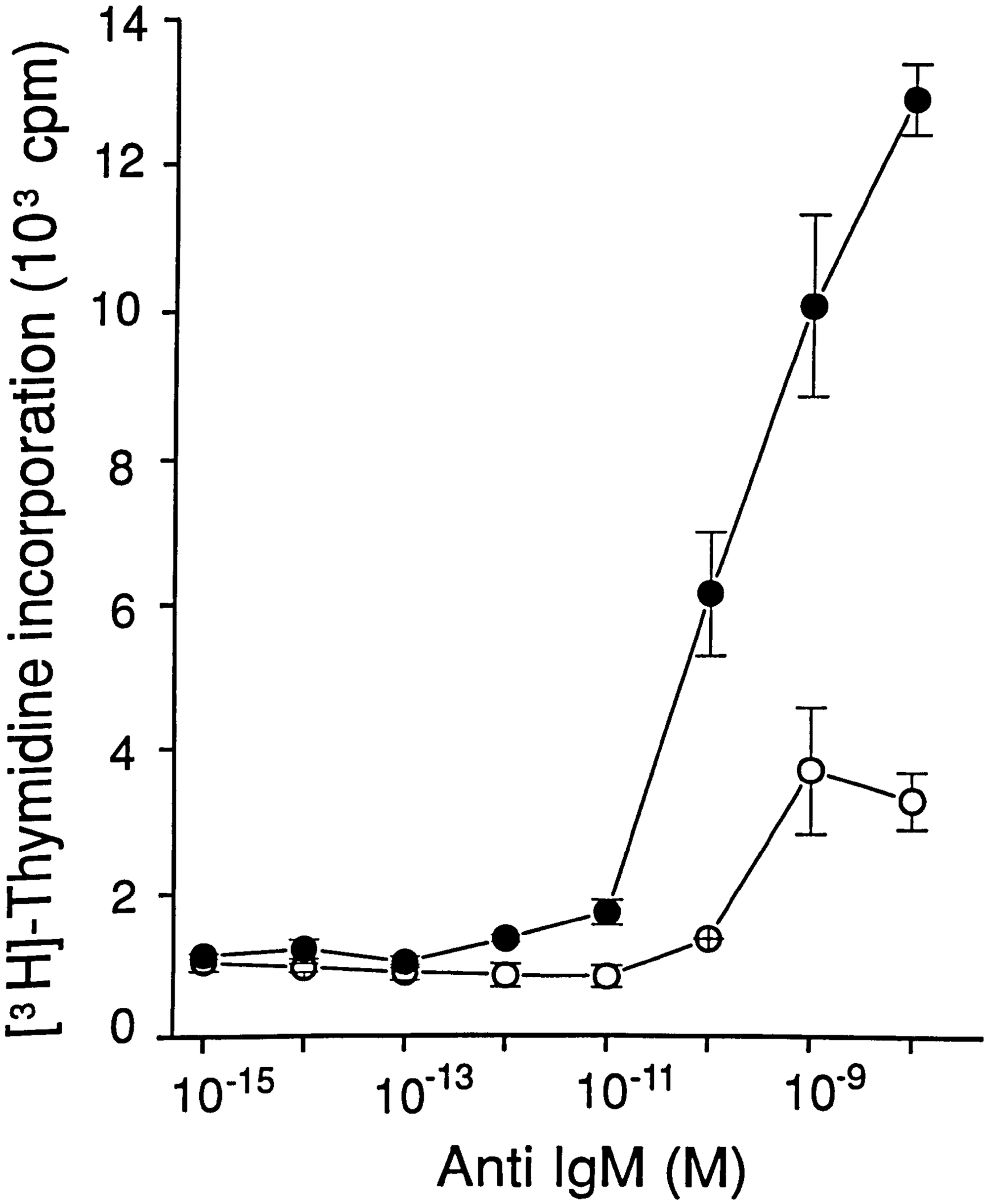


Fig.5-7 CD23 is mitogenic for pre-activated B cells.
The experiment was carried out under exactly the same conditions as in Fig.5-6 except that IL-4 was not added to the cells; $CD32^+/CD23^-$ (○) or $CD32^+/CD23^+$ (●) cells were added. The mitogenic effect of CD23 on the cells that had been pre-activated with anti-IgM is manifested through 2-3 fold enhancement of B cell response to suboptimal concentrations of anti-IgM. The background incorporation was 1000 cpm.

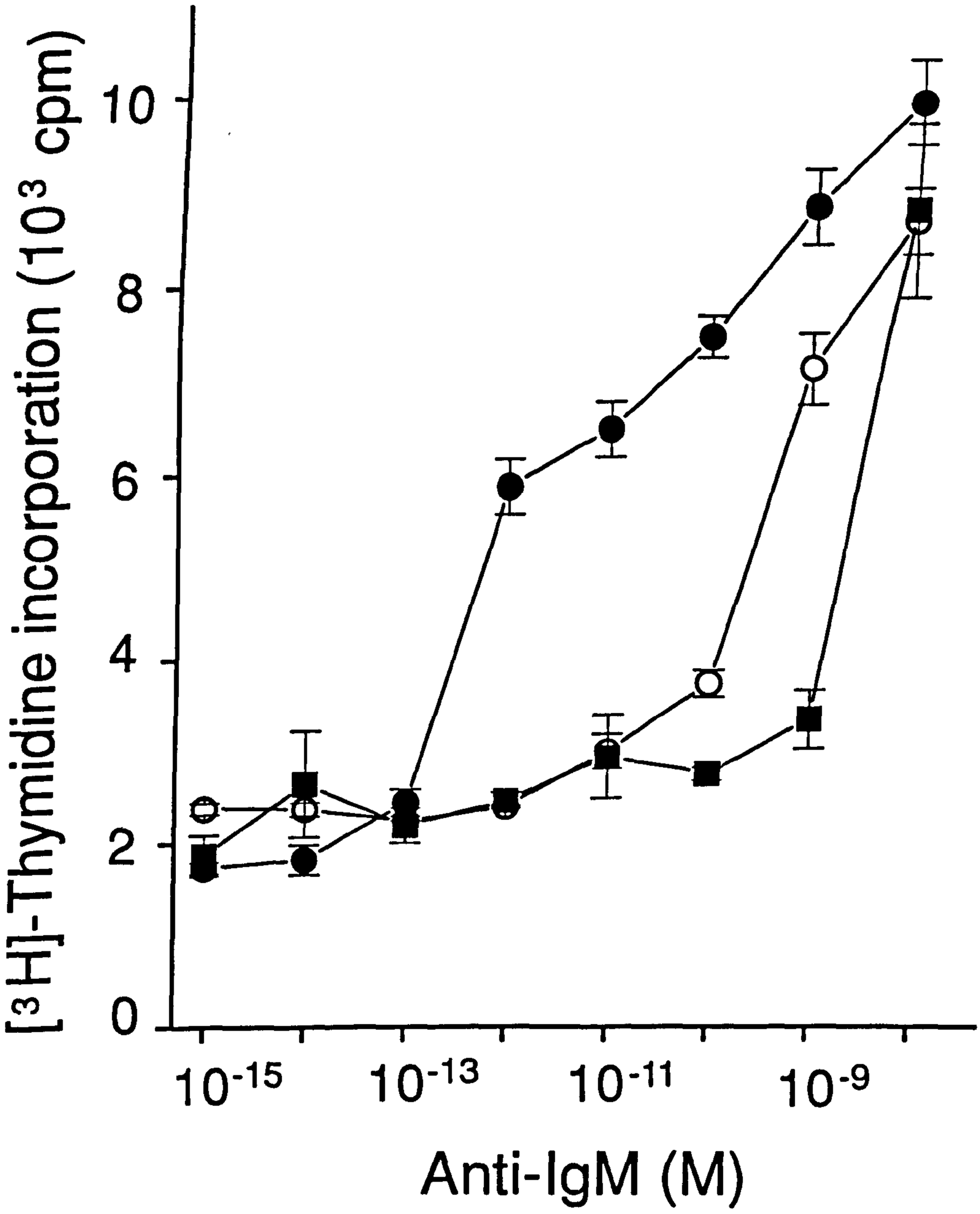


Fig.5-8 Recombinant soluble CR2 inhibits the stimulatory activity of CD23.
B cells were co-cultured with $CD32^+/CD23^-$ (○) or $CD32^+/CD23^+$ (●) cells, or with $CD32^+/CD23^+$ cells in the presence of 2 μg/ml of recombinant CR2 (■). Anti-IgM was added at the incremental concentrations and IL-4 at the concentration of 200 U/ml; the conditions of the experiment were as described above in the text.

5.8. Synergistic interaction of CD19 and IgM lowers the threshold of anti-IgM concentration required for B cell activation

The synergistic effect of crosslinking membrane Ig and CD19 via immobilised monoclonal antibodies to both proteins is manifested through both lowering the threshold of anti-IgM antibody concentration required for the activation of the cell by a factor of 100 and the enhancement of the cell response to optimal antibody concentration by a factor of 2-3 (Carter and Fearon, 1992). In the present study, this was repeated with reasonable accuracy, in that the threshold concentration was indeed decreased from 10^{-10} to 10^{-12} M anti-IgM at least (perhaps even more, but as the increment of concentration increase was 10 it is difficult to extrapolate the exact concentration at which the activation occurs). The enhancement of response in the presence of anti-CD19 antibody HD37 was 2-6 times at the sub-optimal concentration of anti-IgM but only slight at the optimal concentration of the antibody (Fig.5-9).

5.9. CD23 modulates B cell response to CD19 ligation

In order to find out whether the simultaneous co-ligation of CD32 and CD23 on antigen presenting cells such as the fibroblasts in the current assay or FDC *in vivo*, and CD19 and IgM on B cells leads to a further increase in sensitivity of the cell to a given antigen, so that the ligation of even fewer IgM molecules would be required to activate the cell than in the case of either of the two synergistic interactions alone, the tonsillar B cells were incubated with 5 µg/ml ((2.75 x 10^{-8} M) of anti-CD19 antibody and $CD32^+/CD23^-$ or $CD32^+/CD23^+$ fibroblasts. The result is shown in Fig.5-9. In addition to a significant enhancement of proliferation, which cannot be achieved by coligation of either CD19 or CD23 with their respective counterparts for synergistic interaction, the cells also became activated in the effective absence of anti-IgM (concentrations of anti-IgM bel ow 10^{-13} M did not produce any effect on cell proliferation). The effect of coligation of CD19 and CD23 is dependent on the concentration of anti-CD19 antibody used and is detectable at the concentration as low as 100 ng/ml (5.5 x 10^{-10} M), (Fig.5-10).

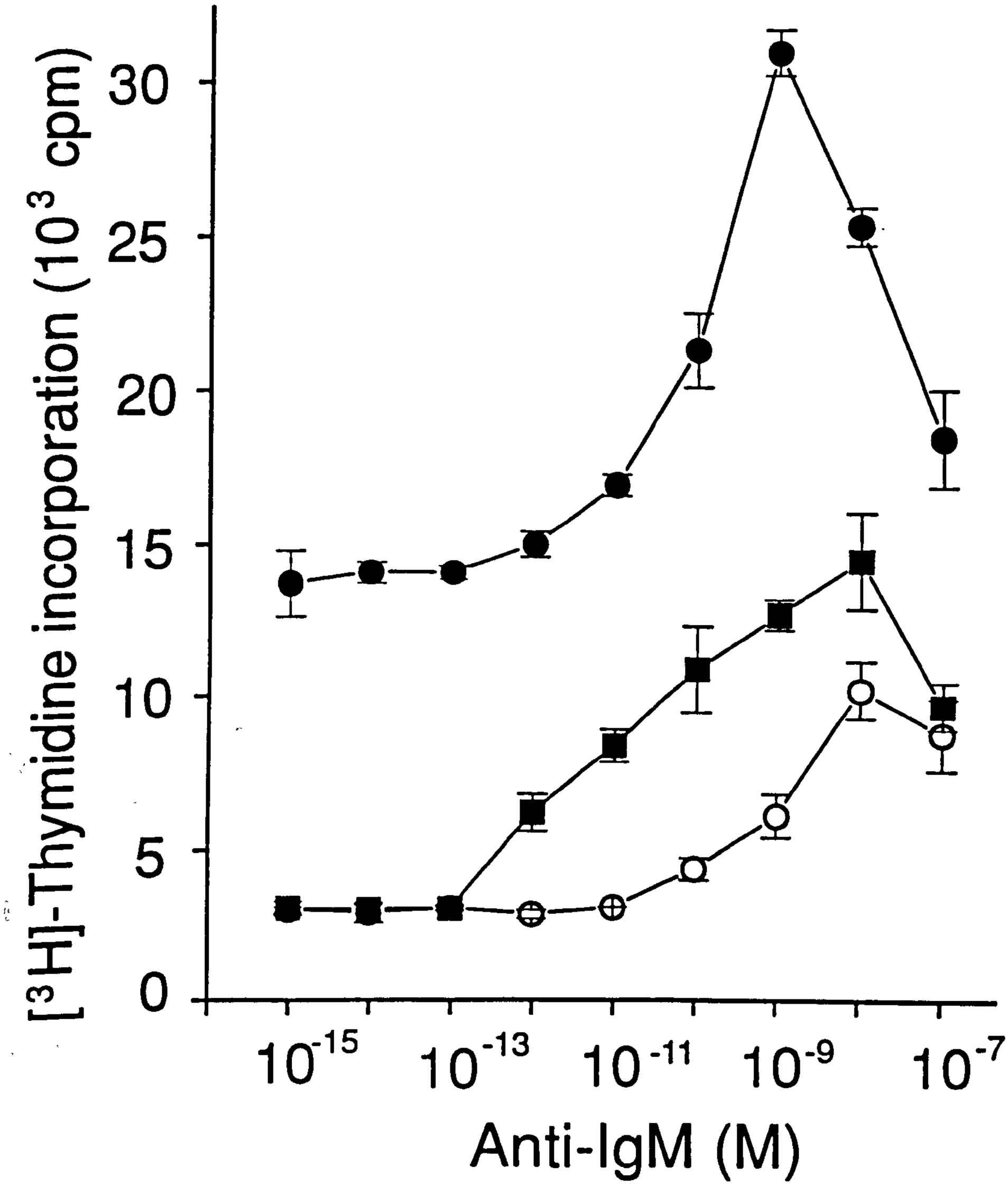


Fig.5-9 CD23 modulates B cell response to CD19 ligation.
B cells were cultured together with $CD32^+/CD23^-$ fibroblast cells alone (○) or in the presence of 5 μg/ml of anti-CD19 antibody HD37 (■), together with anti-IgM at the incremental concentrations and IL-4 at the concentration of 200 U/ml. In the same experiment B cells were also incubated with $CD32^+/CD23^+$ fibroblasts in the presence of anti-CD19 antibody under the same conditions (●). The background incorporation of the label was about 25000 cpm.

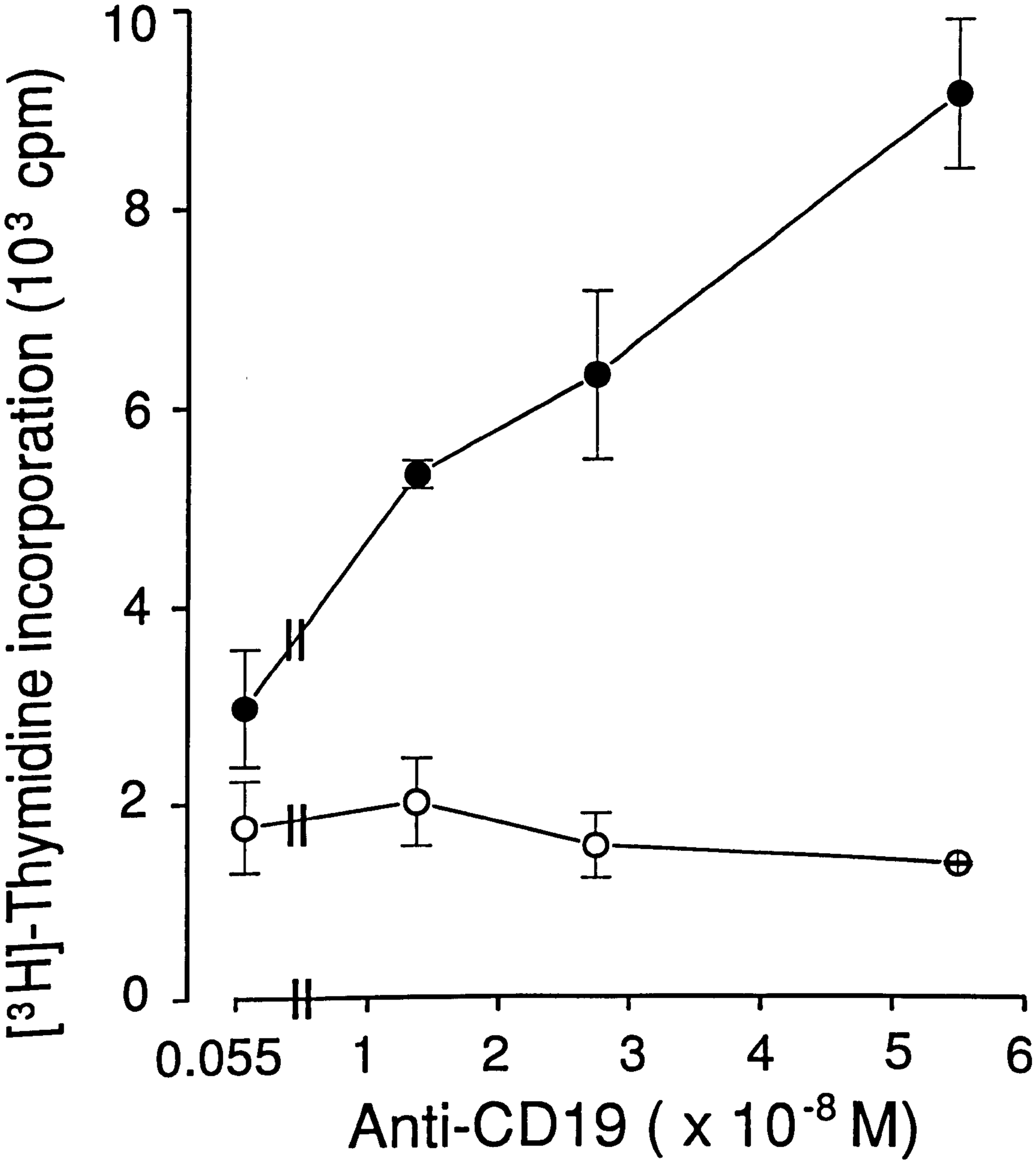


Fig.5-10 Coligation effect of CD19 and CD23 is dependent on concentration of anti-CD19.

The effect of coligation of CD19 and CD23 was tested for a range of anti-CD19 concentration from 5.5 x 10^{-10} to 5.5 x 10^{-8} M; B cells were co-cultured with $CD32^+/CD23^-$ (O) or $CD32^+/CD23^+$ (●) fibroblasts in the presence of anti-CD19 (concentration is indicated on the graph) but not anti-IgM. The background incorporation of the label was 2000 cpm.

5.10 Summary and conclusions

This chapter demonstrates the biological role for CD23-CR2 interaction in the development of B cells. CD23 on antigen presenting cells is able to bind CR2 on B cells, and the consequence of this interaction is a synergistic effect of CD23 and CD32 on IgM-mediated B cell activation and the enhancement of cellular response to the stimulation by an antigen. CD23 also modulates B cell response to CD19 ligation, when combined with IgM ligation, by enhancing the DNA synthesis 2-3 times; moreover, the simultaneous ligation of CD19 by an antibody and CR2 by CD23 was sufficient to activate the cell in the absence of antibody to IgM. The cell response to ligation of CD19 and CR2 is dependent on the concentration of antibody/ligand to CD19, therefore excluding the possibility of direct interaction between CD19 and CD23.

These findings reinforce the role of CD23 in the process of B cell development and add further evidence for physiological importance of CD23-CR2 interaction in the human system. Further studies will be required to clarify the molecular mechanism for the up-regulation of IgE synthesis by CD23-CR2 interaction and the experimental model described in this work could be used to answer that question. Also, the interaction of CD23 and CR2 appears to have different biological outputs with respect to engagement of membrane associated or soluble CD23. The fibroblast cell lines generated in this work should help to explore this aspect of biological function of CD23.

CHAPTER 6: DISCUSSION

6.1. Introduction

The aim of this work was to generate recombinant soluble CD23 that could be used for both biophysical and functional studies of this protein. The recent progress of the knowledge about CD23 made both these studies not only possible but also rather necessary, since it raised a number of new questions and created models that needed further experimental evaluation. The biophysical studies carried out in this work are an attempt to provide the information about the native structure of CD23, as it has been indicated for both murine (Dierks *et al.*, 1993) and human system (Sutton and Gould, 1993) that the structure-function relationship may be very important in the biology of CD23. The functional studies undertaken in this work have largely focused on the interaction of CD23 with CR2 and the role of this interaction in B cell development. The presented data have reinforced the significance of oligomerisation of CD23 for its function and pointed out at the crucial involvement of CD23-CR2 interaction in activation of B cells. It is the objective of this concluding Chapter to provide an interpretation of the obtained experimental data and to discuss the theoretical models that could be proposed on the basis of this and other studies on the subject.

6.2. The native structure of soluble CD23

The full length CD23 protein is a 45 kDa membrane glycoprotein that undergoes proteolytic processing to generate several soluble fragments ranging from 37 to 12 kDa (Delespesse *et al.*, 1989). All of the cleavage fragments have been implicated in one or more biological functions, some of these IgE dependent (Pene *et al.*, 1988; Sarfati *et al.*, 1988, 1992a, 1992b; Capron *et al.*, 1987; Kehry *et al.*, 1989) and the others IgE independent (Gordon *et al.*, 1984, 1985; Liu *et al.*, 1991). These proteolytic fragments differ in the length of the stalk region that is still retained with the lectin domain, with the 25, 16 and 12 kDa fragments lacking the stalk completely. As mentioned before, the stalk region of the protein is thought to be responsible for the self-association of CD23

and this comes from the sequence analysis which provided the evidence for extensive α-helical coiled coil structure formation (Beavil *et al.*, 1992). The protein crosslinking data of both membrane CD23 and its soluble fragments obtained for human CD23 (Beavil *et al.*, 1995) and its murine counterpart (Dierks *et al.*, 1993) confirmed this theoretical prediction in as much that they showed that protein trimers and dimers of CD23 can be formed and detected by crosslinking. As expected, the crosslinking of the full length protein and the 37 kDa fragment had yielded the highest proportion of protein trimer while the 25 kDa fragment produced no detectable trimer and only small amount of dimer. It is possible that the short sequence that comprises 40 amino acid residues and is often called the "neck" region because it links the stalk and the lectin homology domain (see model published by Gould *et al.*, 1991, also Figure 1-2 in this thesis, Chapter 1) is capable of forming α-helical structure as indeed shown for the rat mannose binding protein (Weis and Drickamer, 1994). Thus, the total amount of α-helical structure in CD23 may be even higher then originally predicted in the model by Gould *et al.*, 1991.

The published evidence on the oligomeric structure of CD23, apart from chemical crosslinking, is rather limited. In fact, only one report can be found that describes the biophysical characterisation of a recombinant soluble CD23 fragment expressed in insect cells (Graber et al., 1992). In that report the 37 kDa fragment of CD23 was shown to be monomeric as determined by gel filtration and native PAGE. This protein was able to bind IgE and also to promote the survival of germinal B cells from apoptosis suggesting that the monomeric form of CD23 may still be active, although no reference was made to the affinity of this product for binding to IgE. Several other studies on homologous C-type lectins have been published, but these studies were mostly carried out with either detergent solubilized preparations of the native protein or the recombinant soluble forms comprising the carbohydrate recognition domain (CRD) alone or together with the neck region, thus, providing little information on the contribution of the stalk region to the oligomerisation of the protein. In the case of native protein forms it has been shown that mannose binding protein is an 18-mer, the molecular organization of which is reminiscent of a hexamer of protein trimers (Drickamer and Taylor, 1993). The recombinant fragment of this protein comprising the CRD and the neck region can be crosslinked to form protein trimers and indeed does show this type of structure when crystallised while the

CRD alone forms protein dimers (Weis and Drickamer, 1994; Weis et al., 1991). Other proteins from the type 2 subfamily of C-type animal lectins which are structurally related to CD23 also show tendency of extensive oligomerisation. For example, chicken hepatic lectin, CHL (Loeb and Drickamer, 1987), rat hepatic lectin, RHL, (Halberg *et al.*, 1987) and rabbit hepatic lectin, RbHL, (Andersen et al., 1982) are all hexamers in solution, as shown by chemical crosslinking and/or hydrodynamic studies, but it is worth noticing that the crosslinking of membrane preparations of the former two proteins resulted in the formation of protein dimers and low amounts of trimers rather than high molecular weight oligomers. Interestingly, subtilisin treatment of detergent solubilised CHL that allowed release of a truncated soluble fragment that lacked the transmembrane region, had a dramatic negative effect on the oligomerisation capacity of this protein. This fragment was monomeric as shown by both croslinking and gel-filtration. However, due allowance should be given to the fact that this protein has a rather short stalk region consisted of only two heptad repeats and it is possible that subtilisin treatment has further reduced the length of the available coiled coil structure beyond the point of α-helix stability. Apart from the quoted examples there is no other reported evidence for oligomerisation of soluble CD23.

In this study an approach to this question has been developed that could combine use of several different molecular weight determination methods and in particular ones that could permit the analysis of the oligomerisation state of CD23 at different concentrations of the protein, ranging from 2 µg/ml to 2 mg/ml. These methods included SDS PAGE, native PAGE, HPLC, centrifugation in sucrose gradient, diffusion coefficient measurement, dynamic light scattering, crosslinking of the protein at several different concentrations and circular dichroism measurements. Some of the data obtained by application of these methods are summarised in Table 6-1, together with the data obtained for the two other described recombinant soluble CD23 fragments, one of which is the earlier mentioned 37 kDa fragment expressed in insect cells (Graber *et al.*, 1992; Beavil *et al.*, (1995) and the other is CD23 expressed in mammalian cell line NSO (J. Shi, PhD thesis). One common feature for all three CD23 preparations described in Table 6-1 is the fact that, in spite of being able to form protein trimers that can be detected by crosslinking, none of them yielded a significant amount of protein trimer in any of the employed molecular weight

Table 6-1: Comparison of the structural properties of recombinant soluble forms of CD23 from three different sources.

Structural characteristics	CD23 from insect cells	CD23 from *E. coli*	CD23 from NS0 cells
Protein fragment	"37 KDa"cleave-age fragment	Extracellular portion	Extracellular portion
Theoretical mol. weight	29000	30975	30975
SDS PAGE mol. weight	34000	35000	43000
Native PAGE mol. weight	35000-40000	70000-100000	unknown
Sucrose gradient	unknown	55000/32000	unknown
Superdex-75 HPLC	39000	65000/39000	87000/44000
CD spectrum	25 % α-helix 74 % β-sheet 1 % random	15 % α-helix 56 % β-sheet 29 % random	unknown
Crosslinking	Tm/Mm/Dm at 0.05 mg/ml	Mm/Dm/Tm at 0.002 mg/ml; Tm/Dm at 0.05 mg/ml;	Tm at 0.05 mg/ml
Glycosylation	o-glycosylated	unglycosylated	1 N-glycan, 2-3 O-glycans (not shown)
Degradation fragments	33, 29, 25 KDa	25 KDa mainly, traces of 29 KDa	33, 29, 25, 16 KDa

determination methods. A limited exception to this is the *E. coli* generated protein which showed the presence of a low amount (up to 20 %) of what might be trimeric fraction in the HPLC experiments and native PAGE and also displayed a tetramer-like organisation at the concentration of 2 mg/ml and above, in the dynamic light scattering assay. On the other hand, the protein dimer is easily detectable in most of the applied experiments, at least in the case of *E. coli* and NSO expressed protein, often mixed with monomer rather than existing in a homogenous state. The molecular weight of the dimer of *E. coli* expressed CD23 (55000) was calculated from Swedberg's equation on the basis of measured sedimentation and diffusion coefficients. This is deemed to be the most rigorous way of calculating the native molecular weight of proteins, as it takes into consideration the hydrodynamic parameters that define the behaviour of non-spherical proteins in the solution. The expected molecular weight of the dimer of unglycosylated extracellular domain of CD23 is 60000.

6.3. Concentration dependent oligomerisation of CD23

An apparent discrepancy between the results obtained by chemical crosslinking that detected protein trimer as the predominant fraction and HPLC and sucrose gradient centrifugation experiments that have identified protein dimer as the major fraction has led to the hypothesis that CD23 or at least its soluble part may exist as an equilibrium of the three protein forms, i.e. monomer, dimer and trimer. To test this hypothesis several experiments were performed and the obtained evidence indeed supports this possibility. First it was observed that HPLC profile of CD23 shows the presence of more than one protein form, mainly dimer and monomer and small amount of trimer (Fig.3-17 in Chapter 3) and the same is true for the profile obtained on centrifugation of CD23 in sucrose gradient (Fig.3-19 in Chapter 3), but in this case no protein peak corresponding to the trimer was detected. These experiments were carried out at the concentration of CD23 from 0.2 to 1.33 mg/ml and it was observed that four-fold increase in protein concentration (i.e. from 0.33 to 1.33 mg/ml) produced a noticeable difference in the ratio dimer/monomer, in favour of the dimer (Fig.3-17, Chapter 3). Therefore, it appears that there may exist a concentration dependent equilibrium, at least between monomer and dimer.

Further evidence in support of such a possibility comes from the crosslinking experiments performed at three different protein concentrations of CD23. When crosslinked at the very low concentration (2 µg/ml) CD23 mostly remains a monomer but significant amount of dimer begins to appear (Fig.3-13, Chapter 3). As the further increase of the concentration of the crosslinker did not affect the yield of the formed dimer (not shown in this figure) these conditions were considered optimal for maximum efficiency crosslinking of CD23 at a concentration of 2 µg/ml. If the hypothesis of concentration dependent oligomerisation of CD23 is to hold then one could expect that the addition of unlabelled CD23 to the low concentration of labelled protein should result in the improved yield of the dimer and perhaps trimer upon crosslinking, at least for as long as the concentration of the unlabelled protein is not excessive compared to the concentration of the crosslinker. The obtained result shown in Fig.3-14 in Chapter 3 does indeed support this assumption, as the yield of both dimer and trimer of CD23 increases with the addition of small amounts of unlabelled CD23 (lanes 2-5) but decreases again with further increase of the amount of the unlabelled protein added (lanes 6-9), as the consequence of the crosslinker no longer being in excess. On the other hand, when crosslinked at the concentration of 50 µg/l (25 times higher concentration than in the previous experiment) CD23 gives entirely different pattern; the protein trimer is the major form with some dimer but no monomer present (Fig.3-15, Chapter 3). Therefore, it is apparent that under these conditions CD23 can be almost completely converted to the trimeric form and this is consistent with the results obtained for a similar fragment of CD23 published elsewhere (Beavil et al., 1995). Further still increase in concentration of the protein did not produce conclusive evidence as the significant amount of the protein was intermolecularily crosslinked in a structurally non-specific manner to form high molecular weight protein aggregates of CD23.

Two further experiments demonstrated the concentration dependent oligomerisation of CD23, both performed at high protein concentration. First, the native PAGE revealed that CD23 migrates in the non-denaturing gel as a single although rather diffuse protein band (Fig.3-11, Chapter 3). Since one of the features of the electrophoresis gels is concentrating rather than diluting the samples this can be an efficient method to study the effect of high protein concentration on the oligomerisation state and homogeneity of protein

preparations. A sample of CD23 at the concentration of 1 mg/ml (20 µl) was loaded onto the native PAGE gel with the gradient of polyacrylamide concentration 5-20 % and the electrophoresis was performed as described in Chapter 2. From the dimensions of the excised gel slice containing the protein band stained with Coomassie blue it was measured that the volume of the gel containing CD23 was not greater than 15 µl, thus allowing for a further increase rather than decrease of protein concentration during the electrophoresis. As can be seen in Fig.3-11, Chapter 3, there appears to be only one major protein form that migrates in the gel with a rate that falls between the migration rates of BSA monomer (Mr = 66000) and BSA dimer (Mr = 132000).

Although the main purpose of the above experiment was to determine the molecular homogeneity of CD23 preparation at high protein concentration and no attempt was made to measure the actual molecular weight of the native protein, this experiment is rather illustrative in demonstrating that at this concentration CD23 is not a monomer as it would appear very unlikely that CD23 monomer which is only 30 kDa migrates as a 70-100 kDa protein, even allowing for the fact that the migration of proteins in native gels is rather anomalous, as it is based on the criteria other than those for denaturing gels. However, it is possible to make a more precise estimate of the molecular weight of proteins by native PAGE if gels with gradient of polyacrylamide concentration are used spanning a wide enough range of concentrations to include sufficiently high concentration of the polylinker that will prevent the further migration of the protein due to the small size of the pores. In other words, in such gels the migration rate of proteins will be initially dependent on their charge and size but as they approach the higher concentration of the gel the effect of charge diminishes and the size of the protein becomes the limiting factor for the migration rate. The final migration distance of a certain protein in such gels after they have been overrun for several hours can be considered proportional to the molecular weight of the protein. In the case of CD23 a native gel with 3-30 % gradient of polyacrylamide was prepared and the gel run for 16 h. BSA (a mixture of monomer and dimer) was used as an internal molecular weight marker. Fig.6-1 (this Chapter) shows that the major molecular form of CD23 appears to be a dimer as it runs approximately like the BSA monomer the molecular weight of which is 66000 (theoretical molecular weight for CD23 dimer is 60000). Just about detectable in this figure there appears a

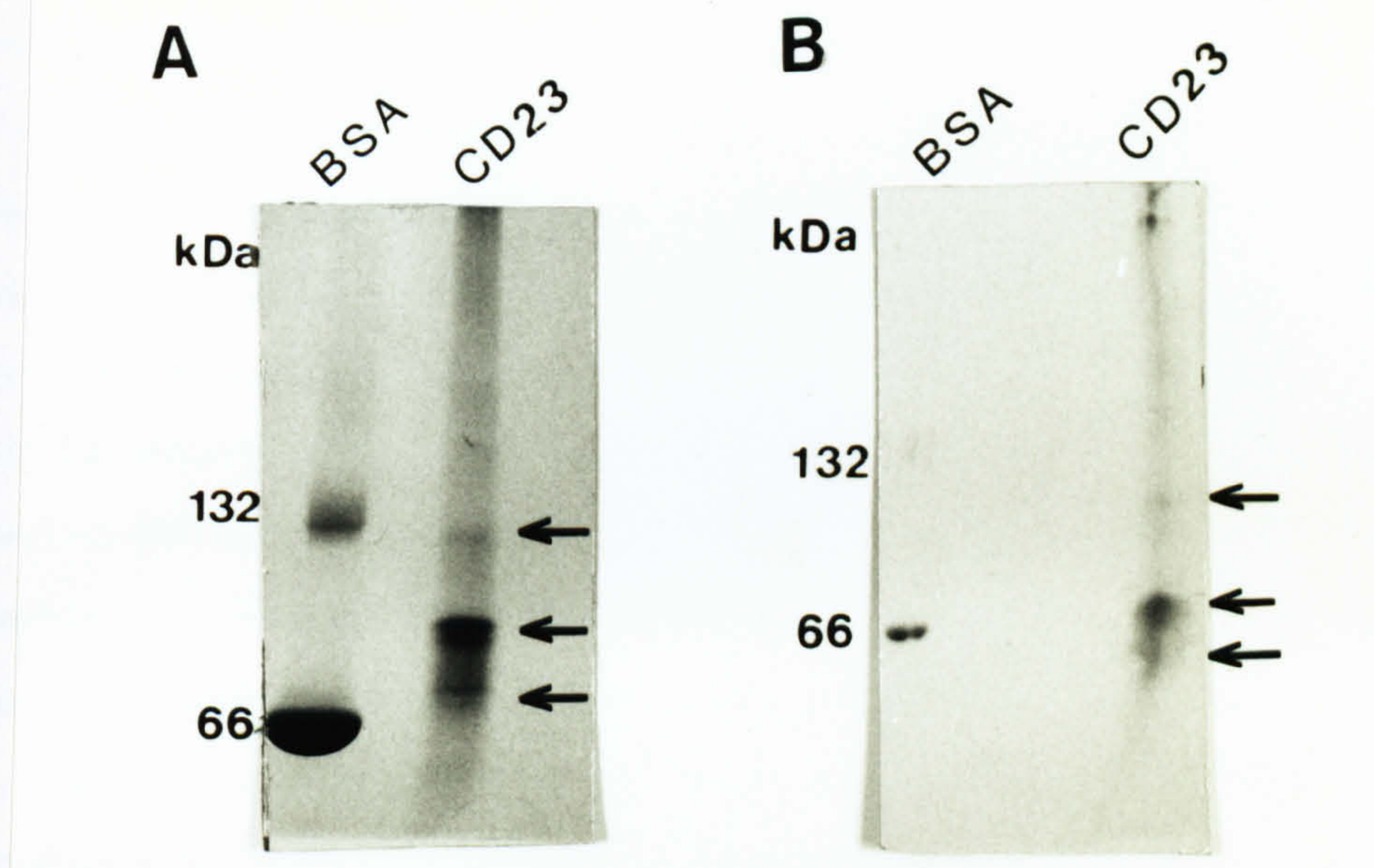


Fig.6-1 Native PAGE of CD23 on 3-30 % polyacrylamide gradient gel.
CD23 (20 µg was applied on to 3-30 % native polyacrylamide gel and the electrophoresis performed for 7 (A) or 16 (B) h. The positions of different CD23 forms (likely to represent protein trimer, dimer and monomer) are marked; the internal molecular weight standards were BSA monomer (66000) and BSA dimer (132000).

larger form of CD23 which runs ahead of BSA dimer and ... is ... likely to be the trimer of CD23, which has the theoretical molecular weight of 90000.

The second experiment performed at the high protein concentration (2 mg/ml) was dynamic light scattering. From the obtained data it can be seen that CD23 at this concentration behaves as a monodisperse system (although one particular measurement gave significant polydispersity factor and it was exempted from the analysis). The interpretation of the data can be summarised as follows: the negligible polydispersity factor means that a possible contribution from minor components to the average value of the Stokes radius is less than 15 % (according to the manufacturer's manual); this reflects in the allowed experimental error for the estimated molecular weight of up to 20 %. The estimated molecular weight for CD23 was 126000 which falls close to the theoretical value for the protein tetramer (120000). This is somewhat unexpected in view of the previous observations that have indicated the protein dimer as the dominant form and the trimer as the minor fraction (native PAGE, also HPLC for lower concentrations of the protein). It is unlikely that this represents an experimental error in the estimation of the molecular weight of the trimer for at least two reasons: firstly, a 40 % error would exceed the maximum allowed error given for the equipment's apprehension of "negligible" polydispersity factor, and, secondly, the native PAGE experiment which was carried out at similar (slightly lower) concentration of the protein indicated that the trimer represents only a minor fraction while the dimer is the dominant form. However, it is possible that the apparent tetramolecular structure of CD23 reflects a higher level association state of CD23, i.e. dimer of a dimer.

6.4 Higher order of self-association of CD23

Higher level oligomerisation is common for other C-type lectins, notably for MBP (Drickamer and Taylor, 1993), CHL (Loeb and Drickamer, 1987), RHL (Halberg *et al.*, 1987) and RbHL (Andersen *et al.*, 1982), but in each of these cases the transmembrane region is thought to be responsible for the interaction. Such evidence (though not on the same structural basis) already exists for CD23 as well, in the form of crosslinking data performed by Beavil *et al.*, 1995, who showed that a crosslinker with sufficiently long

"spacer arm" could crosslink CD23 to form protein hexamers, as well as all the lower intermediate forms. This was possible not only in the case of the 37 kDa fragment which contains a large portion of the stalk region but also in the case of 25 kDa fragment which completely lacks the stalk region and is not expected to oligomerise through α-helix formation (although the possibility exists that the neck region may be capable of forming low amount of this type of structure on its own, as could be predicted by analogy to MBP (Weis and Drickamer, 1994). The ability of the lectin domain to self-associate has been demonstrated for MBP, for which it was shown that CRD domain alone is capable of forming protein dimers although it does not comprise any coiled coil structure (Weis and Drickamer, 1994). Therefore, it appears that the lectin domains of CD23 can interact on a higher level of oligomerisation to generate dimers of the dimer or indeed the dimer of a trimer although the later could not be detected in the described light scattering experiment. Nevertheless, the two parameters associated with the criteria for the distinction between the monomodal and bimodal system, the base line (represents the fit of the regression analysis applied) and the sum of squares, SOS (represents the amount of residual noise or error associated with the autocorrelation function), both have the border values that indicate possible "contamination" with a component of higher molecular weight, presumably, protein hexamer. This type of self-association of CD23 or other C-type lectins seems to be possible only in solution and not on the cell membrane, since the crosslinking experiments with the membrane associated proteins consistently failed to detect any oligomeric form higher than trimer (Loeb and Drickamer, 1987; Beavil *et al.*, 1995). A simple explanation for this discrepancy could be that the membrane associated protein lacks the required level of freedom of movement which could enable the protein dimers or trimers to dock against each other in a sterically feasible manner.

There may be several questions arising from the possibility of higher level of self-association of CD23. Here are only two. How stable are these homocomplexes? What possible role (if any) this type of oligomerisation might have? The answer to the first question would require identification of the domains and residues involved in this interaction, but the data presented in this work and the evidence from elsewhere published reports allow for a general conclusion that the interactions involved must be weak because very few methods seem to be appropriate to detect them. Chemical crosslinking is one of

these methods but its disadvantage lies in the fact that it does not reflect the actual dynamic state of the system. The answer to the second question may be even more difficult. There is no evidence in the published literature that would suggest a possible physiological role for this type of oligomerisation. However, one suggestion could be made on the basis of results obtained in a study of the effect of recombinant soluble CD23 described here on the internalisation of IgE immune complexes mediated by CD23 on B cells (S. Karagianis, PhD thesis). This study had shown that addition of low amount of soluble CD23 enhances rather than inhibits the uptake of IgE and a possible mechanism by which this could happen may be the higher level self-association of CD23 and an increase in avidity of the receptor/ligand interaction.

6.5. Model for concentration dependent oligomerisation of CD23: a summary

Based on the experimental evidence presented in Chapter 3 and the interpretations of the experimental data as described above, a schematic model could be proposed depicting various stages and levels of oligomerisation and their dependence on the concentration of the protein. The model is designed on the basis of the data obtained for soluble CD23 and may not hold true or may require corrections for the cell membrane associated CD23. At low protein concentration (2 µg/ml; 6.7 x 10^{-8} M) CD23 is monomer with some dimer present. At 50 µg/ml (1.67 x 10^{-6} M) CD23 can form protein trimer that can be detected by crosslinking with some dimer as an intermediate. Since no evidence that the trimer of CD23 is the dominant form (at this or even higher protein concentration) was found by conventional molecular weight measurement methods it is likely that there exist an equilibrium between the dimer and the trimer, as well as between the dimer and the monomer. The difference is, however, in the affinity of the association which in the former case appears to be low and in the later case higher. The transition of the dimer to a trimer seems to be the limiting factor in the oligomerisation of CD23. At the concentration up to 1.33 mg/ml (4.4 x 10^{-5} M) there is still a low amount of the protein monomer present but the proportion of the trimer increases up to 20 %. Further increase in concentration (> 4.4 x 10^{-5} M) eliminates the monomer completely but does not significantly increase the proportion of the trimer. At the concentration of 2 mg/ml (6.7 x 10^{-5} M) and above there appears to begin higher level of association of CD23 in the

form of protein tetramers and hexamers. The ratio tetramer/hexamer may resemble the equilibrium between the dimer and the trimer. Therefore, the critical concentrations seem to be 10^{-6} M for the transition of monomer to dimer, 10^{-5} M for the transition of the dimer to trimer and above 10^{-5} M for higher order oligomerisation (formation of tetramer and hexamer). The limiting factor for the formation of the higher oligomers, i.e. trimers and hexamers that are thought to resemble the native structure of CD23 is the low association constant for the formation of the protein trimer. Schematically, this could be depicted as shown in Fig.6-2.

6.6. The interaction of CD23 with CR2

The interaction of CD23 with CR2 was discovered in 1992 by Aubry *et al.*, who showed that native CD23 when incorporated in fluorescent liposomes binds specifically to several B cell types and that this could be blocked by several antibodies to CR2 antigen. The binding of CD23 to CR2 was shown to be Ca^{2+} dependent and inhibited by the monosaccharide derivative fucose-1-phosphate, thus, implicating the lectin type nature of the interaction. This finding, together with the functional studies that followed,has solved several questions concerning the biological functions of CD23 and offered new interpretations of the existing experimental models. Probably most of all, it provided CD23 with a ligand for its lectin functions that have been somewhat ignored ever since the discovery that oligosaccharidesare not required for the interaction of CD23 with IgE (Vercelli *et al.*, 1989).

The first report on the interaction of CD23 and CR2 (Aubry et al., 1992) pointed out that recombinant soluble CD23 failed to bind CR2 in contrast to the intact protein and the explanation for this was the possibility that oligomerisation of the protein may be a requirement for the interaction. Indeed, the recombinant CD23 used in quoted report was a baculovirus expressed 37 kDa CD23 fragment, which has been previously shown by gel filtration to be monomeric (Graber *et al.*, 1992). In the current work it was demonstrated that soluble CD23 expressed in *E. coli* is mainly a dimer, but also that there is an equilibrium between the dimer and trimer. It was also shown that this recombinant preparation of CD23 is able to bind CR2 on RPMI 8226 cells, as demonstrated by its

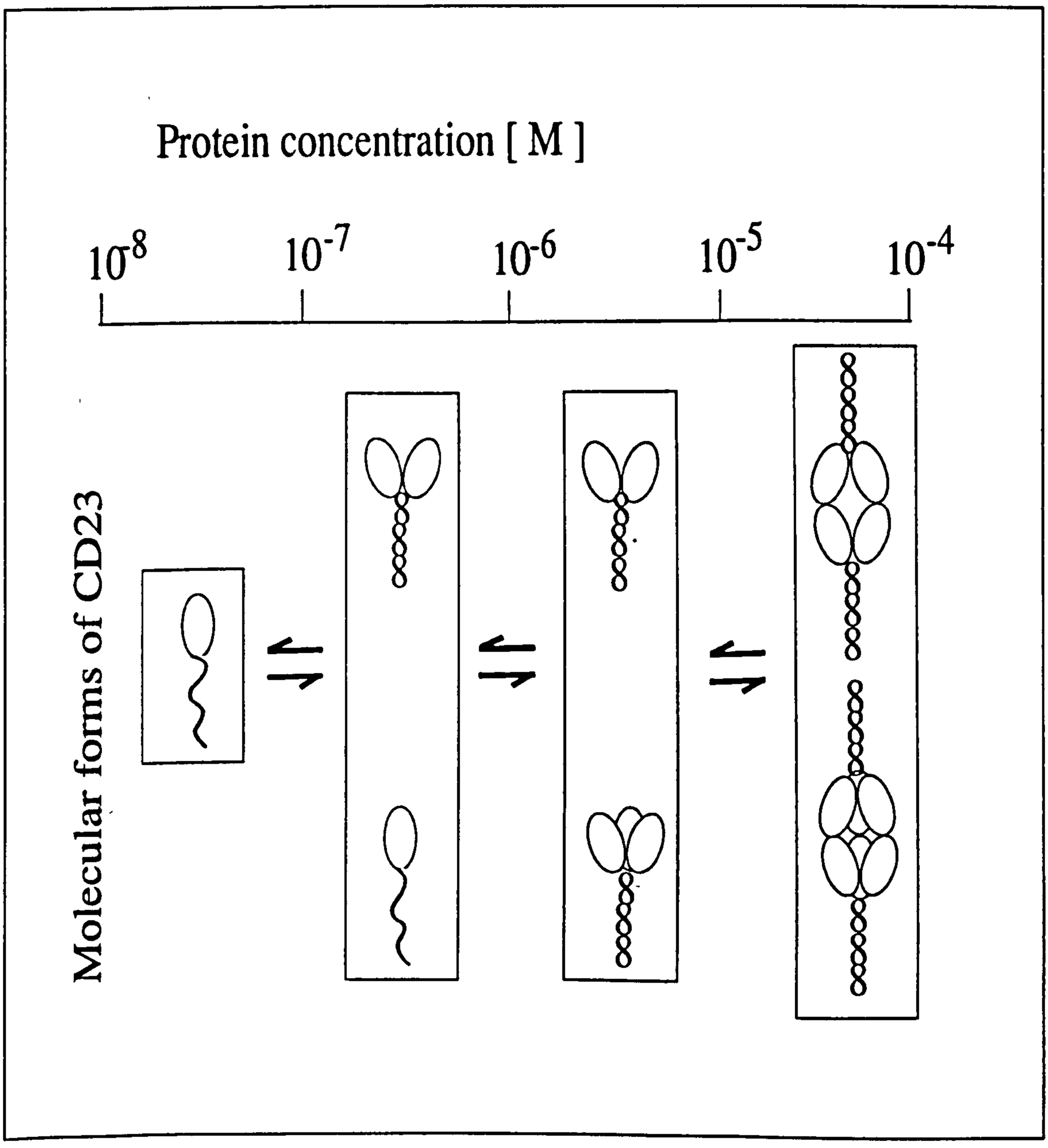


Fig.6-2 Concentration dependent oligomerisation of CD23.
This schematic model for concentration dependent oligomerisation of soluble CD23 depicts several concentrations that are thought to be critical for transition between the different molecular forms of CD23; although it is based on experimental observations this model should be considered as an approximation rather than an accurate reflection of the true oligomerisation state of CD23 at the given concentrations.

capacity to inhibit the binding of native CD23. However, from the inhibition pattern obtained by varying the concentration of the recombinant CD23 in the assay and the incubation time it appears that the affinity of the recombinant protein is somewhat lower than that of the native protein.

The binding of recombinant soluble CD23 to CR2 on RPMI 8226 cells has been subject of another study which failed to detect the interaction (Ishihara *et al.*, 1994). The reason for this might be the inappropriateness of the experimental assay used (Western blotting type binding assay as opposed to the cell binding assay employed in this work); also the authors of the quoted report failed to demonstrate that under their experimental conditions the full length CD23 was able to bind CR2, (i.e. the positive control in the experiment) and no description of the recombinant CD23 fragment used was offered. It is clear that soluble CD23 fragments that lack most of the stalk region (such as the 25 kDa fragment) are not able to oligomerise in the same manner as the intact protein or the extracellular part, which might significantly influence the affinity for the interaction with CR2. However, it is true that 25 kDa fragment of CD23 appears to be the most stable intermediate in the proteolytic fragmentation pattern of CD23 (Cairns and Gordon, 1990) and probably the most relevant for the functional studies. In this work no attempt has been made to test all the degradation products of CD23 for their capacity to bind CR2, but the SPR data on 16 kDa CD23 fragment, which consists of the lectin domain alone with no extra sequences on either side (J. Shi, PhD thesis) still exhibited measurable although somewhat weaker binding of CR2 (not shown). Therefore, it is likely that the ability of soluble CD23 fragments to bind CR2 will depend on their respective sizes and oligomerisation capacity.

6.7. The duality of the binding site for CD23 on CR2

The recombinant CR2 secreted by insect cells was shown to bind weakly to recombinant CD23 (Ka was of the order of magnitude 10^{-5}-10^{-6} M). The binding site on CR2 for the interaction with CD23 appears to be dual: the carbohydrates from the SCRs 5-8 (in particular the carbohydrate at the Asn position 370) as well as the peptide backbone of SCRs 1 and 2 contribute to the total binding. As the glycosylation of CR2 may indeed

vary from one cell type to another it is to be expected that some cell types will bind CD23 better than the others. In this work, a recombinant CR2 expressed in insect cells was used and shown to be able to bind CD23. Although the recombinant protein isolated from insect cell supernatants was confirmed to be glycosylated it is likely that the structure of the attached oligosaccharide significantly differed from those in animal cells. The insect cells largely do not synthesise complex type carbohydrates, but rather truncated high mannose type oligosaccharide. However, few examples demonstrate that other monosaccharides may be included as well, notably fucose attached to the primary glucosamine residue (Wathen *et al.*, 1990). Moreover, evidence does exist that glycoproteins synthesised in insect cells can bind fucose-specific lectins (Russell and Consigly, 1985), a relevant observation with respect to apparent carbohydrate binding specificity of CD23 in the interaction with CR2 (Aubry *et al.*, 1992). Insect cells are also capable of O-type glycosylation but not sialiation of galactose residues (Wathen *et al.*, 1990); this leaves them accessible for interaction with lectins. Again, this was an important consideration with regard to second reported carbohydrate-binding specificity of CD23 (Kijimoto-Ochiai *et al.*, 1994; Kijimoto-Ochiai and Uede, 1995). Thus, based on the available published evidence, it could not be predicted in advance whether recombinant CR2 expressed in insect cells would possess sufficiently correct carbohydrate structures for binding CD23.

According to the obtained result it might be concluded that the glycosylation of CR2 in insect cells was sufficiently appropriate for binding CD23, although the measured affinity constant (4.6×10^{-5} M) was rather low and it cannot be excluded that the observed binding could be partly or entirely mediated through the glycosylation independent binding site within the SCRs 1 and 2. It is not possible to determine the actual contributions of these two binding sites to the total binding since it would be difficult to predict whether the carbohydrates on CR2 may affect the secondary structure of SCRs 1 and 2 (and in that way influence their contribution to binding), and also, whether these two SCRs may influence the glycosylation of the rest of the protein, due to the conformational requirements. Thus, if the binding site for CD23 on CR2 molecule is split, the key questions to be answered in the future experiments are: how do the two epitopes relate to each other, are they mutually dependent and how do they interact with CD23

molecule. The preliminary evidence on the stoichiometry of the interaction of CD23 with its other ligand, IgE, indicates the possibility that CD23 itself may possess two binding sites for IgE (J. Shi et al, submitted for publication), thus giving further credibility to the theory that the interaction of CD23 and CR2 may involve dual binding sites on both proteins.

6.8. CD23 is a co-stimulator of B cell activation

The secondary immune response to antigen in the differentiation pathway of B lymphocytes is a requirement for affinity maturation of these cells which then give rise to either antibody secreting plasma cells or memory B cells. The ligation of the surface immunoglobulin (sIg) alone would normally require a relatively high concentration of antigen to activate the cell, a difficulty that the B cell resolves by simultaneous engagement of both the sIg and the other membrane proteins such as CD19 (Carter and Fearon, 1992) and CR2 (Carter et al., 1988). The synergistic interaction between these and other membrane proteins and the antigen receptor leads to increased responsiveness of the cell to given antigen and in that way modulates the sIg mediated B cell stimulation. However, little is known about natural ligands for these proteins on antigen presenting cells such as FDC. FDC are non-lymphoid cells present in secondary lymphoid tissues and are generally thought to play an important role at several stages of B cell activation and maturation. In addition to providing the antigen in immobilised and unprocessed form, the FDC are implicated in co-stimulation of the pre-activated B cells (Burton *et al.*, 1993) and in survival and proliferation of the selected germinal center (GC) B cells (Lindhout *et al.*, 1993; Kosco *et al.*, 1992). The means by which the FDC can achieve this are at least three triple: (i) antigen specific dependent clonal selection of GC cells through matching the expressed antibody isotype with the antigen on FDC, (ii) direct cell contact with B cells through adhesion molecules, and (iii) secretion of stimulatory factors that promote the survival and clonal expansion of antigen specific cells.

A particularly remarkable phenotypic feature of FDC is high level expression of CD23. One possible role for CD23 on FDC has already been suggested, based on the evidence that a soluble fragment of this protein in synergy with IL-1α provides the rescue signal

for GC B cells that would otherwise undergo apoptosis (Liu *et al.*, 1991). In the current work, evidence was sought for the role of membrane associated CD23 on FDC in the context of B cell activation, with particular regard for the possibility that the CD23-CR2 adhesion pair may be involved in FDC-B cell interactions. A hypothesis has been previously proposed, which envisages the CD23-CR2 interaction as one of the critical sites of the interaction, alongside the FcγR-antibody/antigen-sIg interaction, between the antigen presenting and B cell (Sutton and Gould, 1993). The model also predicts a synergistic type interaction between CD32 (FcγRII, the low affinity receptor for IgG) and CD23, by analogy to the synergistic effects of CD19 or CR2 co-ligation in combination with the antigen receptor ligation. To test this hypothesis and to further clarify the role of CD23 in B cell activation and differentiation, a fibroblast cell line that expresses both CD32 and CD23 was generated and could in that respect mimic FDC in B cell. proliferation assay. Highly purified resting B cells from human tonsils were stimulated with incremental concentrations of anti-IgM antibody, in the presence of mitomycin treated fibroblast cells and IL-4. The sensitivity of B cell to the ligation of sIg was increased by a factor of 10-100 if the B cells were co-cultured with $CD32^+/CD23^+$ fibroblasts as compared to $CD32^+/CD23^-$ cells. CD23 also displayed a significant response enhancement effect in the presence of sub-optimal concentration of anti-IgM and this was particularly obvious when the cells were cultured in the absence of IL-4. Therefore, in addition to synergistic interaction with CD32 that leads to increased sensitivity of the cell for a given antigen, CD23 is also mitogenic for pre-activated cells. This is consistent with a previous report that had suggested such a role for CD23 based on the capacity of purified intact protein to stimulate the phorbol-ester pre-activated cells (Cairns and Gordon, 1990), and in fact, further extends this suggestion by demonstration of mitogenic activity of the cell membrane associated CD23.

6.9. CD23-CR2 interaction is responsible for B cell stimulatory activity of CD23

CR2 was the obvious choice of the candidate for the role of counter-structure for CD23 on B cell, not only because it has been already known as the second ligand for CD23 but also because CR2 itself displays stimulatory effect on B cell response when co-ligated with sIg (Carter *et al.*, 1988; Luxembourg *et al.*, 1994). Besides that, this protein has been

implicated in the rescue of GC cells from apoptosis upon engagement with a subset of monoclonal antibodies, thus providing a rationale for the capacity of soluble CD23 to promote the survival of these cells (Bonnefoy *et al.*, 1993). Recombinant soluble CR2 expressed in insect cells was added to the culture of B cells and $CD32^+/CD23^-$ or $CD32^+/CD23^+$ fibroblasts, together with incremental concentrations of anti-IgM and IL-4. As shown in Fig.5-8, the stimulatory effect of CD23 is completely diminished in the presence of recombinant CR2. In fact, soluble CR2 not only displaced its membrane associated counterpart from the interaction with CD23 but also exhibited somewhat inhibitory effect on sIg mediated stimulation of the cells, presumably a consequence of its engagement in interactions with other surface proteins that may be activated by ligation of sIg and its inability to transduce the signals into the cell because of the lack of intracellular domain that is responsible for signalling through CR2. Thus, CD23 must now be added to the list of CR2 ligands (C3d, EBV) and antibodies that are capable of triggering B cell activation through this receptor, particularly in combination with antigen receptor ligation. A very recent report (Dempsey *et al.*, 1996) revealed a dramatic effect of C3d attachment to a model antigen in stimulating the immune response *in vivo*; only 3 copies of C3d attached to the antigen enhanced the response in immunised mice by a factor of 10000. This was shown to be mediated through CR2 which is the receptor for this complement fragment and crosslinking of the antigen receptor and CR2 by C3dg may be considered as a molecular bridge between the innate and acquired immunity. CD23 binding to CR2 further reinforces this notion and in fact is a unique example of a direct interaction between the acquired immune and complement system without participation of C3dg as a crosslinker. Thus, CR2, together with CD19, emerges as a major modulator of B cell response; it interacts with the antigen/receptor complex through C3dg, it participates in cellular contact between the B and the antigen presenting through CD23 and finally, it associates with CD19 and modulates the signalling through this molecule.

6.10. CD23 modulates B cell response to CD19 ligation

The simultaneous engagement of CD19 on B cells by a monoclonal antibody and CR2 by CD23 on fibroblast cells significantly enhanced B cell response to IgM ligation (Fig. 5-9).

Moreover, it was shown that the engagement of these two membrane proteins was already sufficient to activate the cell in the absence of antibody/antigen. Thus, it is possible that simultaneous engagement of CR2 (with CD23 and possibly other ligands) and CD19 (with unknown ligand), both of which are members of closely associated membrane protein complex that has great signal transducing capacity (Fearon and Carter, 1995) is sufficient alone to activate the cell. Although the ligand for CD19 and its possible expression on B cells and FDC are not yet known, the role of CD19 as the modulator of cellular response to antigen receptor ligation is well established, as shown by both *in vitro* (Carter *et al.*, 1988) and *in vivo* studies (Sato *et al.*, 1995). Moreover, CD19 appears to be a regulator of other membrane proteins; for example, engaging of CD19 by a monoclonal antibody induces the binding of B cells to the interfollicular stroma of tonsils via integrin α4/β1 (VLA-4) (Behr and Schriever, 1995). In turn, the VLA-4 adhesion pathway, together with the LFA-1 integrin dependent pathway, is shown to prevent apoptosis of GC B cells (Koopman *et al.*, 1994), the role which is closely associated with soluble CD23. Indeed, the only published report that places CD19 and CD23 into the same perspective showed that in addition to other factors, an anti-CD19 antibody was a potent inhibitor of IL-4 promoted CD23 synthesis and surface expression in B cells, and that this inhibitory effect could be overridden by engagement of CD40 (Gordon *et al.*, 1991). If this is so, then one could speculate that the engagement of CD19 on B cells results in the inhibition of CD23 expression which in turn decreases the probability for homotypic B cell interactions through CD23 and CR2 and favours the stimulatory FDC-B cell contact.

6.11. Concluding remarks and implications for future studies

This and other reports further delineate the difference in biological function of membrane associated CD23 and its soluble fragments and also emphasize the duality of the nature of this protein. Its role as the low affinity receptor for IgE and the modulator of IgE synthesis has been well documented. The role of CD23 as a B cell differentiation marker and an autocrine growth factor began to emerge with the discovery of its second ligand, CR2. This work has demonstrated that the membrane associated CD23 is co-stimulatory for B cells and that this function is mediated by two types of interactions, the synergistic interaction with CD32 and the adhesion type interaction with CR2. The later interaction

appears to form a link between the two distinct biological activities of CD23 with respect to binding IgE and CR2, since it has been shown previously that this interaction, in addition to having other possible functions also up-regulates IgE synthesis (Aubry *et al.*, 1992; Bonnefoy *et al.*, 1995). It would be therefore of considerable interest to study the molecular mechanism for the regulation of IgE synthesis by CD23-CR2 interaction. It has been observed previously that in contrast to its soluble fragments, the membrane associated CD23 down-regulates IgE synthesis (reviewed by Sutton and Gould, 1993). It is very likely that the engagement of CD23 with CR2 is responsible for the increase in IgE synthesis and the mechanism by which this is achieved may well resemble the one described in this work in the context of B cell activation. In other words, it is possible that ligation of CD23 on an antigen presenting cell with CR2 on IgE committed B cell, in the presence of IL-4, may lead to increase of IgE production and amplification of the cellular response to the allergen. Also, the *in vitro* crosslinking of IgE and CR2 with oligomeric soluble CD23 which has been demonstrated in this work may have important functional consequences in the context of allergic response. These and other studies that have been already initiated as the continuation of current work should further clarify the role of CD23 in the biology of B cell.

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